Regulatory Functions of Sialylated Milk Oligosaccharides in Mucosal Immunity

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Andrea Corinne Fuhrer

von

Trubschachen BE

Promotionskomitee

Prof. Dr. Thierry Hennet (Vorsitz)

Prof. Dr. Thomas Brunner

Prof. Dr. Eric G. Berger

Dr. Norbert Sprenger

Zürich, 2010

Table of contents

Summary

Human milk is one of the richest biological fluids. It is considered to be the gold standard of infant nutrition providing essential nutrients for optimal growth and development. Besides nutrition, breast milk helps protect the immunologically immature newborn from enteric infections, the development of allergy, or inflammation of the gastrointestinal tract. This protection is mediated by a variety of soluble and cellular factors in breast milk such as secretory immunoglobulin A, lysozyme, lactoferrin and oligosaccharides. In human milk, oligosaccharides are the third largest component besides lactose and fat. Their structural diversity is high and unique since other mammalian milk oligosaccharides are less complex. The main function of milk oligosaccharides is the prevention of pathogen attachment to epithelial cell surface carbohydrates by acting as soluble surface receptors. However the exact role of milk oligosaccharide on immune development, tolerance and regulation of inflammation is not fully understood.

In this thesis I studied the function of specific milk oligosaccharides, the sialyllactoses, on mucosal immunity. The complexity of mouse milk oligosaccharides is low as it only contains lactose and two forms of sialyllactose, namely 3SL and 6SL. To investigate sialyllactose function, we used α2,3- and α2,6 sialyltransferase-null mice that lack either 3SL or 6SL. Cross-fostering experiments between C57Bl/6 and sialyltransferase-null mice were setup to feed mice with normal or sialyllactose-deficient milk. Neither the exposure to normal, nor to sialyllactose-deficient milk had an effect on the development of mucosal leukocyte populations. However, when adult mice were challenged by dextran sulfate sodium (DSS) in drinking water, mice previously exposed to 3SL-deficient milk were more resistant to colitis than mice exposed to normal or 6SL-deficient milk. The analysis of intestinal microbiota revealed a different colonization pattern depending on the presence or absence of 3SL. A *Ruminococcaceae* species was enriched in the intestine of mice exposed to 3SL during lactation. We correlated the composition of the intestinal microbiota to the severity of DSS-induced colitis by colonizing germfree mice with microbiota from mice fed with normal or 3SL-deficient milk. As anticipated, mice colonized with microbiota derived from mice exposed to 3SL-deficient milk were less susceptible to DSS-induced colitis than mice colonized with normal microbiota.

In conclusion, we demonstrate that the exposure to a single milk oligosaccharide exerts long term effects on intestinal bacterial colonization, as shown by *Ruminococcaceae* specie enrichment. Furthermore we show that the bacterial colonization affects the susceptibility of the host to DSS-induced colitis. In this study we provide evidence that Ruminococcaceae are pro-

inflammatory bacteria that are specifically enriched through 3SL exposure. To examine the exact contribution of *Ruminococcaceae* in the development of DSS-induced colitis further experiments are required in particular the specific enrichment of *Ruminococcaceae* in culture.

Zusammenfassung

Muttermilch ist eines der reichhaltigsten biologischen Sekrete überhaupt. Sie ist das „non plus ultra“ in der Säuglingsernährung, da sie essentielle Nährstoffe für eine optimale Entwicklung des Säuglings liefert. Neben dem Ernähren des Säuglings schützt die Muttermilch den immunologisch unreifen Säugling vor Infektionen, der Entwicklung von Allergien oder Entzündungen im Gastrointestinaltrakt. Dieser Schutz wird durch eine Reihe von löslichen und zellulären Faktoren in der Muttermilch vermittelt. Diese beinhalten sekretorisches Immunglobulin A, Lysozym, Lactoferrin und Oligosaccharide. Die Oligosaccharide sind neben Fett und Laktose die drittgrösste Komponente in der Humanmilch. Ihre hohe Strukturdiversität ist einzigartig in der Säugetiermilch. Die Oligosaccharide in der Muttermilch verhindern das Anheften von Pathogenen an die Epithelzellen der Darmmukosa, indem sie als lösliche Rezeptoranaloge fungieren. Ihr Rolle in der Entwicklung des Immunsystems, der Toleranz und der Regulation von Entzündungprozessen ist jedoch unbekannt.

Während meiner Doktorarbeit untersuchten wir die Funktion von spezifischen Milch-Oligosacchariden, den Sialyllaktosen, im mukosalen Immunsystem. Die Oligosaccharide in der Mausmilch, Laktose und die zwei Formen von Sialyllaktose (3SL und 6SL), weisen nur eine geringe Komplexität auf. Um die Funktion der Sialyllaktose zu untersuchen, verwendeten wir α2,3- und α2,6 Sialyltransferase-defiziente Mäuse, die keine 3SL oder 6SL aufweisen. In sogenanten Fremdpflege-Experimenten zwischen C57Bl/6 und Sialyltransferase-defizienten Mäusen, wurden die Mäuse mit normaler oder Sialyllaktose-defizienter Milch gefüttert. Weder die Fütterung von normaler noch Sialyllaktose-defizienter Milch hatte eine Auswirkung auf die Entwicklung der Leukozytenpopulationen in der Mukosa. Allerdings zeigten adulte Mäuse, die in Präsenz von 3SL-defizienter Milch aufwuchsen eine höhere Resistenz gegenüber einer Dextran Sulfat Sodium (DSS)-induzierten Kolitis im Vergleich zu Mäusen, die mit normaler oder 6SL-defizienter Milch gefüttert wurden. Die Analyse der intestinalen Mikrobiota zeigte ein unterschiedliches Kolonisierungsmuster, abhängig von der Präsenz oder Absenz von 3SL. Die Prägung durch 3SL während der Laktation hatte eine Anreicherung von Ruminococcaceae Spezies im Kolon zur Folge. Durch die Kolonisierung keimfreier Mäuse mit der Mikrobiota von Mäusen, die mit normaler oder 3SL-defizienter Milch gefüttert wurden, konnten wir eine Korrelation zwischen der Zusammensetzung der intestinalen Mikrobiota und der Empfindlichkeit für DSS-induzierte Kolitis feststellen. Mäuse, die die Mikrobiota der mit 3SL-defizienter Milch gefütterten Tiere erhielten, waren weniger empfindlich für DSS-induzierte Kolitis als Kontrollmäuse.

Zusammenfassend zeigen wir anhand der erhöhten Ansammlung von *Ruminococcaceae* Spezies, dass die Prägung durch ein einzelnes Milch-Oligosaccharid langzeitig die Kolonisierung des Kolons beeinflussen kann. Des Weiteren konnten wir zeigen, dass die bakterielle Zusammensetzung die Empfindlichkeit der Mäuse für DSS-induzierte Kolitis beeinflusst. Wir geben in dieser Doktorarbeit Hinweise dafür, dass Ruminococcaceae Spezies eine entzündungsfördernde Wirkung aufweisen können und ihre Anreicherung im Kolon spezifisch auf der Exposition von 3SL basiert. Um den genauen Beitrag der Ruminococcaceae Spezies in der Entwicklung von DSS-induzierter Kolitis zu untersuchen, sind jedoch weitere Experimente erforderlich. Dies beeinhaltet insbesondere die Anreicherung der Ruminococcoaceae Spezies in Kultur.

Abbreviations

3SL	sialyl(α2,3)lactose
6SL	sialyl(α2,6)lactose
CD	Cluster of differentiation
CF	Cross-fostering
DSS	Dextran sulfate sodium
Fuc	Fucose
Gal	Galactose
GALT	Gut associated lymphoid tissue
Gb3	Globotriaosylceramide
GIT	Gastrointestinal tract
Glc	Glucose
GlcNAc	N-acetylglucosamine
GM1/3	Mono/tri-sialotetrahexosylganglioside
HPAEC	High performance anion exchange chromatography
IBD	Inflammatory bowel disease
IgA	Immunoglobulin A
IL	Interleukine
IRAK	Interleukin 1 receptor-associated kinase
IRF3	Interferon-regulatory factor 3
Ικκ	IκB kinase
M cell	Microfold cell
MyD88	Myeloid differentiation primary response gene (88)
NALT	Nasopharynx associated lymphoid tissue
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid

NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	nucleotide-binding oligomerization domain
PPARγ	Peroxisome proliferator-activated receptor gamma
RT	Reverse transcription
Sia	Sialic acid
SIGGR	Single immunoglobulin IL-1R-related molecule
SL	Sialyllactose
T reg	T regulatory cell
TGF-β	Transforming Growth Factor beta
Th1	T helper 1
Th2	T helper 2
TIRAP	toll-interleukin 1 receptor domain containing adaptor protein;
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alfa
TOLLIP	Toll interacting protein
TTGE	temporal temperature gradient gel electrophoresis
Wt	Wild type

Introduction

Human milk

Human milk is considered to be the „gold standard“ in infant nutrition by providing optimal nutrients for normal growth and development. These nutrients include macromolecules such as carbohydrates, lipids and proteins and micronutrients such as vitamins and minerals (Newton, 2004). Apart from nutritional benefits, human milk contains multiple bioactive and immunomodulatory components. They provide active and passive immunity in the early months and years of life when the neonate's immune system starts to fully develop (Lönnerdal, 2010). The importance of human milk is illustrated in developing countries where breastfeeding has shown protective effects on mortality rate. Not only were death rates decreased, but improvements of cognitive and psychosocial development were also documented (Anderson et al., 1999; Labbok et al., 2004).

Bioactive molecules in milk

Components of the adaptive immune system

The most recognized protection from mother to the child is transplacental passage of immunoglobulins during pregnancy followed by secretory immunoglobulin A (IgA) (Hugot et al.) in milk during lactation (Hanson, 1961). IgA is produced by plasma cells in the mammary gland that originate from primed B cells from the small intestine or the bronchial tree (Halsey et al., 1983). Receptor mediated transport of dimeric IgA through the mammary epithelial cell results in secretion in milk (Hanson, 1998). Through breastfeeding IgA enters the gastrointestinal tract without being digested, binds to enteric pathogens and thus inhibits their attachment to the epithelial layer. The inability of the neonate to produce IgA in the first few weeks of life, is thus compensated (Hanson et al., 1991) (Fig. 1 and 2).

In addition to antibody mediated immunity, cellular immunity is transferred from mother to child. Depending on the stage of lactation, multiple leukocyte types are present in milk (Goldman, 1993). The most common are macrophages and neutrophils. Lymphocytes, mostly T cells, are also present. Macrophages for example express activation markers and are suggested to influence lymphocyte function in infants by releasing immunmodulatory cytokines. Additionally they contain engulfed IgA, which is most likely released on contact with bacteria in the gut inhibiting microbial attachment to the epithelial layer (Brandtzaeg, 2003). Activated T cells in milk are hypothesized to compensate for the immature function of neonatal T cells and assist their

maturation (Eglinton et al., 1994). In addition to local effects, systemic effects of milk lymphocytes were suggested, as they were found in the intestinal mucosa and local lymph nodes of the offspring (Hanson and Korotkova, 2002) (Fig. 2).

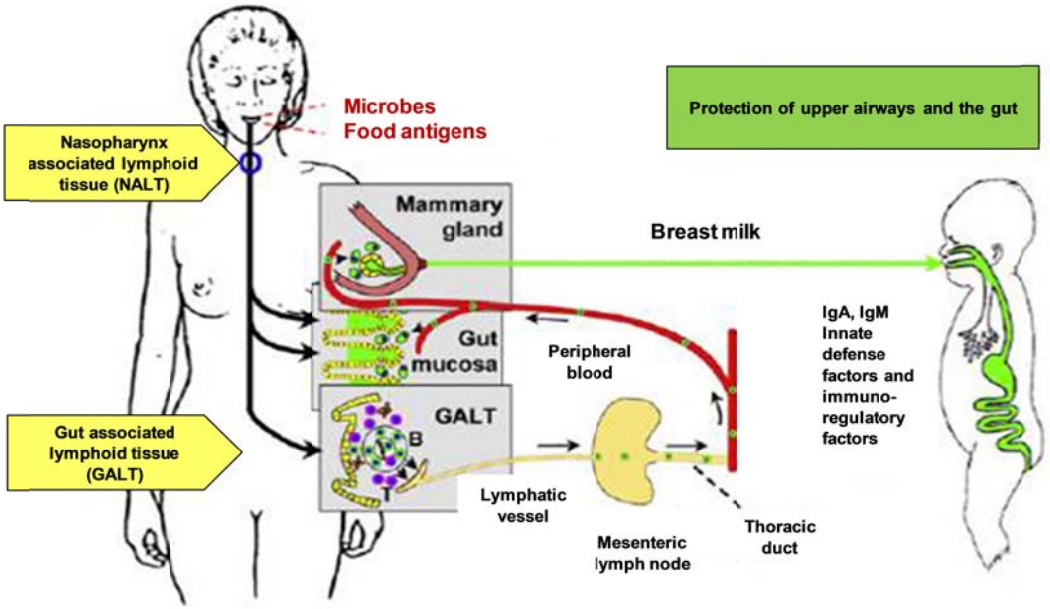


Figure 1: Integration of mucosal immunity between mother and the newborn.

Integration of mucosal immunity between mother and the newborn, with emphasis on migration of effector B cells from gut associated lymphoid tissue (GALT) via lymph and peripheral blood to the lactating mammary gland. Distribution (arrows) beyond the gut of precursors for IgA plasma cells is crucial for local production and subsequent occurrence in breast milk of IgA and smaller amounts of IgM antibodies specific for enteric and airway antigens (microorganisms and exogenous proteins). The breast-fed infant will receive relevant secretory antibodies directed against the microbiota colonizing its mucosae and is therefore better protected against infections in the gut and the upper airway (green areas). NALT: Nasopharynx associated lymphoid tissue (modified from (Brandtzaeg, 2010)).

Multifunctional milk components

In addition to components of the adaptive immune system transferred from the mother to the child, other soluble agents with antimicrobial functions are also present in breast milk (Lönnerdal, 2010). Lactoferrin is a ferric iron-binding glycoprotein present in high concentration in milk (Lönnerdal and Lyer, 1995). It competes with bacteria for available ferric iron and thus limits bacterial growth. An additional control on bacterial load is the lysozyme. It destroys the bacterial cell wall by cleaving the cell wall component peptidoglycan (Labbok et al., 2004).

Heavily glycosylated milk mucins or lactadherin inhibit binding of certain pathogens to epithelial cells (Schroten et al., 1992). Small lipids such as free fatty acids and monoglycerides are produced by enzymatic digestion of triglycerides in human milk. They protect the neonate from viral infections by destroying enveloped viruses (Isaacs, 2001). Finally, hormones and growth factors are additional bioactive molecules found in milk that modulate the gastrointestinal tract and are to a certain extent also absorbed into systemic circulation (Goldman, 2000) (Fig. 2).

Immunomodulatory agents

Human milk contains a great number of cytokines. These cytokines impact humoral and cellular immunity, macrophage activity and pro- or anti-inflammatory effects (Goldman, 1993). The main source of cytokines is the mammary gland itself, although small amounts are also directly produced from leukocytes in milk. Two anti-inflammatory cytokines that are present in human milk are IL-10 and TGF-β (Goldman et al. 1982). IL-10 dampens Th1 response by inhibition of pro-inflammatory cytokine release (Garofalo et al., 1995). TGF-β is implicated in immune regulation by down-regulating inflammation and improving healing of damaged intestinal cells (Field, 2005; Saito et al., 1993). Pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNFα are also found in milk. However, their activity is reduced by the presence of cognate soluble receptors or receptor antagonists (Field, 2005; Hawkes et al., 2002). Moreover, the higher concentration of the anti-inflammatory cytokine IL-10 in milk suggests an overall anti-inflammatory condition mediated by milk components. Several questions about the physiological role of cytokines in milk remain unanswered. For example it is unclear how cytokines survive transit through the gastrointestinal tract. Other immunomodulatory agents such as a soluble form of Toll-like receptor (TLR) 2 and soluble CD14 might sensitize the innate immune system during bacterial colonization (Labeta et al., 2000; LeBouder et al., 2003). TLR2 is activated by Gram-positive bacteria. The soluble form found in milk might prevent extensive activation upon bacterial recognition during colonization and thus prevent diseases such as necrotizing enterocolitis (LeBouder et al., 2006). CD14 plays a pivotal role in recognition of microbial cell wall components of both Gram-positive and Gram-negative bacteria. The high concentration of CD14 in milk indicates its role in regulating immune responses of neonates during bacterial colonization (Labeta et al., 2000) (Fig. 2).

Milk oligosaccharides

Human milk oligosaccharides are the third largest solute besides lactose and fat. By definition, oligosaccharides are free carbohydrates comprised of between three to ten monosaccharides covalently linked by a glycosidic bond (Kunz et al., 2000). Milk oligosaccharides are indigestible by the infant gut and therefore are not used as nutrients (Engfer et al., 2000; Gnoth et al., 2000). Milk oligosaccharides were initially believed to be accidental functionless by-products of active glycosyltransferases in the mammary gland during lactation (Newburg et al., 2005). However the structural homology of free oligosaccharides to epithelial cell surface glycans suggested they are functionally active. Indeed, one of their main functions lies in their ability to serve as receptor homologues of cell surface receptors, thereby inhibiting bacterial attachment to the cell surface (Kunz et al., 2000) (Fig. 2).

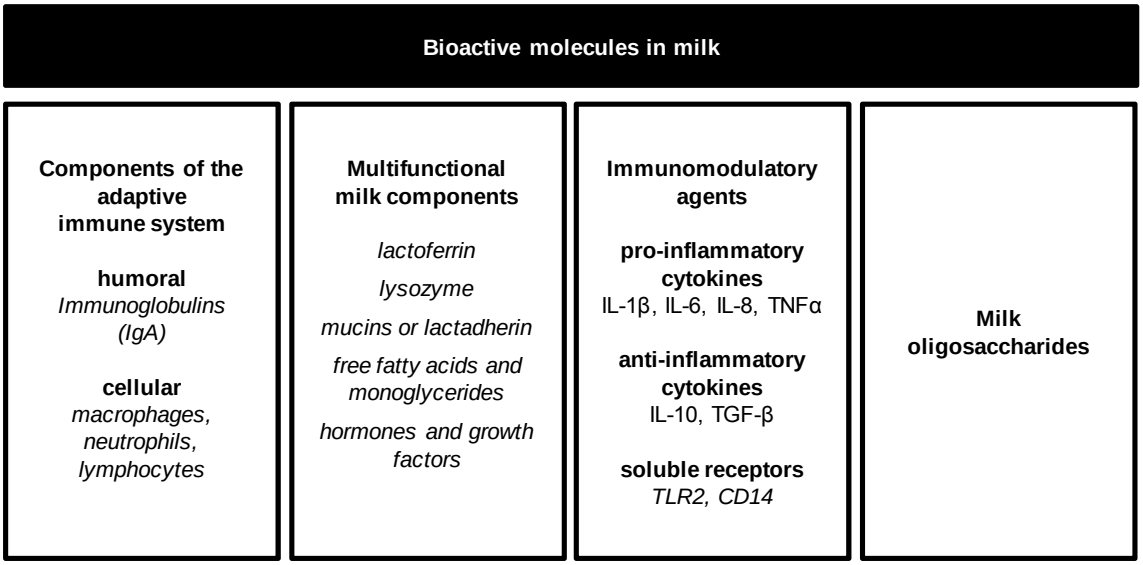


Figure 2: Bioactive molecules in milk.

Bioactive molecules in milk can be divided into four functional groups: components of the adaptive immune system, multifunctional milk components, immunomodulatory agents, milk oligosaccharides.

Milk oligosaccharide structure

One liter of human milk contains 5-10 g of oligosaccharides placing this fraction within the three largest components of human milk along with proteins and lipids (Kunz Rudloff 2002). The composition and variability of milk oligosaccharides is mediated by a set of glycosyltransferase enzymes, whose expressions are regulated in the mammary gland. The sugar monomers include glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acid (Sia). Most milk oligosaccharides have the disaccharide lactose (Galβ1,4Glc) at their reducing end. Elongation at the non-reducing end is performed by an enzymatic transfer of sugar monomers. Characteristic elongations contain repetitive units of the disaccharide N-acetyllactosamine (Galβ1,3/4GlcNAc). The lactose or N-acetyllactosamine backbones are further modified by α2,3-, α2,6 sialylation or α1,2-, α1,3-, or α1,4 fucosylation. The modification of lactose by either sialic acid or fucose form the smallest oligosaccharide fraction such as 2'fucosyllatose or 3'fucosyllatose and 3'sialyllactose or 6'sialyllactose respectively (Kunz et al., 2000) (Fig. 3).

Milk oligosaccharides are divided into neutral and acidic oligosaccharides, depending on the absence or presence of sialic acid (Gopal and Gill, 2000). Approximately 200 different structures of neutral and acidic oligosaccharide have been detected to date (Mehra and Kelly, 2006). The concentration and structure of milk oligosaccharides between individuals depends on the secretor status, the ABO blood group type and the Lewis blood group type (Newburg, 2000; Viverge et al., 1990). The called "nonsecretor" mothers lack active copies of the gene coding for fucosyltransferase FucT-II and therefore cannot synthesize fucose(alpha)1,2 epitopes such as 2'fucosyllactose. The prevalence of nonsecretor mothers in Europe is approximately 20% (Viverge et al., 1990). Another fucosyltransferase termed FucT-III which is the only fucosyltranferase that attaches fucose to N-acetylglucosamine in an α1,4 linkage is found in "Lewis-positive" but not "Lewis-negative" individuals. Thus milk oligosaccharides depend on Lewis blood group and secretor status and are therefore different in their structures (Thurl et al., 1997).

The concentration of milk oligosaccharides varies strongly during lactation (Miller et al., 1994). High concentrations of oligosaccharides are detected during early lactation with levels 5-10 times lower towards weaning (Mehra and Kelly, 2006). Human milk is very unique in the high content and complexity of milk oligosaccharides, especially when compared to domestic mammals such as cows, sheep, goats and horses (Urashima et al., 1997). A pronounced

difference is seen in the neutral fraction of non-human milk oligosaccharides. This fraction contains only few oligosaccharides that are fucosylated and is dominated by oligosaccharides linked to Gal or GlcNAc. Moreover, uniquely found in non-human milk are attachments of α1,3Gal or α1,3GalNAc at the non-reducing terminus (Urashima et al., 2001). Furthermore, in the acidic fraction of non-human milk oligosaccharides two distinct forms of sialic acid exists, N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) whereas in human milk only Neu5Ac exists. The loss of Neu5Gc in humans is caused by a mutation in the human gene coding for CMP-Neu5Ac hydroxylase, an enzyme that converts the sugar nucleotide CMP-Neu5Ac to CMP-NeuGc (Boehm and Stahl, 2007; Brinkman-Van der Linden et al., 2000; Urashima et al., 2001).

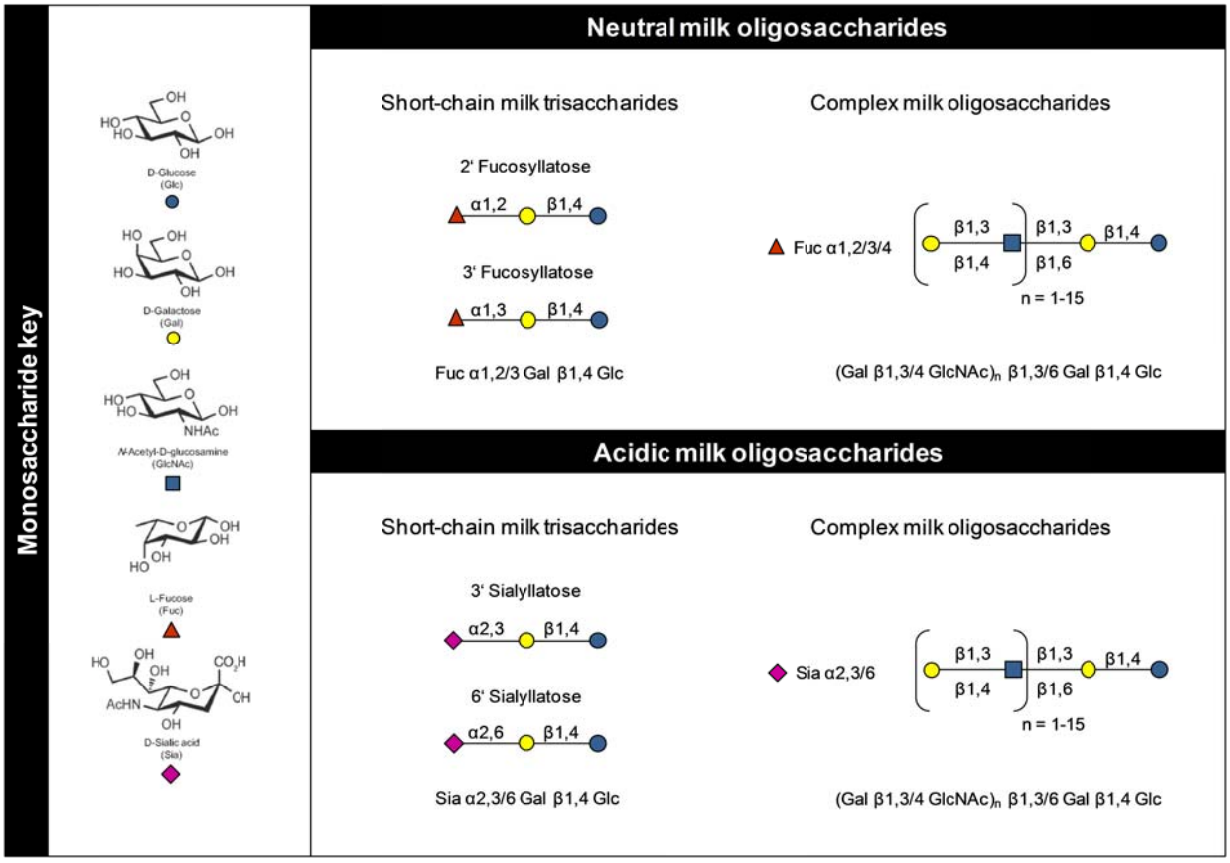


Figure 3. Structural compostition of milk oligosaccharides.

Milk oligosaccharides are classified as neutral and acidic oligosaccharides. They contain short-chain trisaccharides, e.g.,sialyllactose or fucosyllactose, or complex high-molecular-weight glycans. (Modified from Bode, 2009).

Biological effects of human milk oligosaccharides

Prebiotics

The definition of a prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of a limited number of bacteria in the colon, and thus improves host health (Gibson and Roberfroid, 1995). Human milk oligosaccharides in breast milk are considered prebiotics. Most are indigestible due to lack of luminal enzymes able to cleave most glycosidic linkages in the gastrointestinal tract (Engfer et al., 2000; Gnoth et al., 2000). However, bacteria in the colon express glycosidases and metabolize human milk oligosaccharides (Hill, 1995; Sela and Mills, 2010). Studies have shown that initial feeding with milk oligosaccharides changes the microbiota towards a predominant population of gram-positive nonsporulating bacilli, *Bifidobacteria*, and lactic acid-producing organisms (*Lactobacilli*).

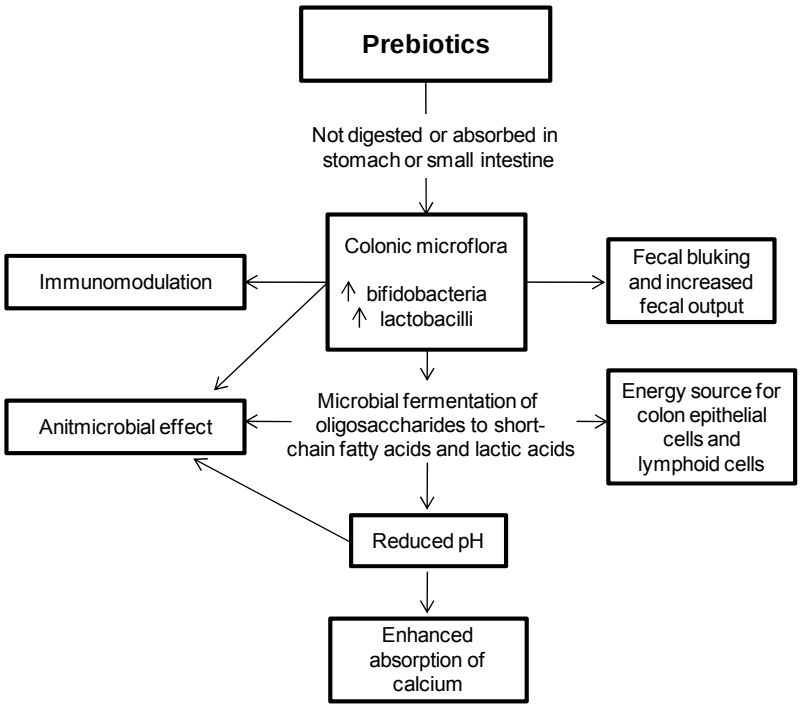


Figure 4: Potential effects of prebiotics.

Prebiotics are best known for selectively increasing the probiotic bacteria *bifidobacteria* and *lactobacilli*. These probiotic bacteria influence fecal texture and fecal output. Furthermore, they modulate immune responses by for example increasing IgA production (see probiotic section). The fermentation products of the probiotic bacteria such as short-chain fatty acids and lactic acids further improve host's health by delivering energy and by creating a hostile environment for potential pathogens by acidification and a decreased pH. (modified from (Sherman et al., 2009).

Non-breastfed infants show an adult-type microbiota with predominance of *Enterobacteriaceae*, *Clostridium*, and *Bacteroides* (Harmsen et al., 2000). Health promoting effects of prebiotics are indirectly mediated through the resident *Bifidobacteria* that produce short-chain fatty acids and lactic acid by fermentation, thus creating an acidic milieu. This milieu increases the absorption of calcium and magnesium and creates a hostile environment for potential pathogens (Abrams et al., 2005; Sherman et al., 2009). Potential effects of prebiotics are summarized in Fig. 4 (Sherman et al., 2009).

Anti-adhesive effects

Adhesion to the epithelial cell surface is the first step of a bacterial infection. Therefore virulence of many pathogens depends on their ability to bind to a host epithelial surface. This adhesion is often mediated by bacterial lectins, glycan-binding proteins. Structural homology between milk oligosaccharides and cell surface glycans suggests that milk oligosaccharides are used as soluble decoys that prevent pathogen adhesion and thereby infection (Newburg et al., 2005; Sharon, 1996). This was illustrated by the binding of *Campylobacter jejuni*, one of the major causes of diarrhea worldwide, to soluble 2'-fucosyllactose (Cravioto et al., 1990). Fucosylated milk oligosaccharides vary among mothers due to genetic polymorphisms of the responsible fucosyltransferases. The incidence of *Campylobacter* induced diarrhea was shown to be inversely related to the amount of soluble 2'-fucosyllactose present in mother's milk (Morrow et al., 2004). Ex vivo experiments confirmed an inhibitory effect of fucosylated milk oligosaccharides on the attachment of *Campylobacter* to the intestinal mucosa (Ruiz-Palacios et al., 2003). Table 1 shows additional identified milk oligosaccharide species that inhibit pathogenesis of bacterial and viral infections.

Table 1: Pathogen inhibition through human milk glycans (modified from (Newburg, 2009))

A great number of human milk glycans were identified to inhibit pathogens. These glycans include free oligosaccharides (fucosylated and sialylated), mucins (O-glycosidic-linked high-molecular-mass glycoproteins in the membranes surrounding milk fat globules), glycosaminoglycans such as chondroitin sulfate (long unbranched polysaccharides consisting of a repeating disaccharide unit) and glycoshingolipids (sulfatide, Gb3: Globotriaosylceramide; GM1/3: mono/tri-sialotetrahexosylganglioside).

Glycan	Pathogen	Reference
Oligosaccharide fractions	*Streptococcus pneumonia*	(Andersson et al., 1986)
	Enteropathogenic *Escherichia coli*	(Cravioto et al., 1990)
	Listeria monocytogenes	(Coppa et al., 2003)
Fucosylated oligosaccharides	*Campylobacter jejuni*	(Ruiz-Palacios et al., 2003)
	Vibrio cholerae	(Ruiz-Palacios et al., 2003)
	Escherichia coli Stable toxin	(Newburg, 1999)
Sialyllactose	Cholera toxin	(Idota et al., 1995)
	Enteropathogenic *Escherichia coli*	(Stins et al., 1994; Virkola et al., 1993)
	P. aeruginosa	(Devaraj et al., 1994)
	Aspergillus fumigatus conidia	(Bouchara et al., 1997)
	Influenza virus	(Gambaryan et al., 1997; Matrosovich et al., 1993)
	Polyomavirus	(Stehle et al., 1994)
	Helicobacter pylori	(Mysore et al., 1998)
Mucin	S-fimbriated *E. coli*	(Schroten et al., 1992)
Chondroitin sulfate	HIV	(Newburg et al., 1995)
Sulfatide	HIV	(Viveros-Rogel et al., 2004)
Gb3	Shiga toxin	(Newburg et al., 1992)
GM1	Labile toxin, cholera toxin	(Otnaess et al., 1983)
GM3	Enteropathogenic *Escherichia coli*	(Idota and Kawakami, 1995)

Impact on intestinal epithelial cells

Milk oligosaccharides have been shown to exhibit direct effects on glycosylation patterns of epithelial cell surface glycans. An *in vitro* study using Caco-2 cells treated with 3'sialyllactose (3SL) showed reduced cell surface expression of α2,3 and α2,6 linked sialic acid residues (Angeloni et al., 2005). This glycome-modifying effect reduced binding of enteropahtogenic *Escherichia coli* by 50%. The cause of reduced sialic acid residues was found to be a lower expression level of certain sialyltransferases. Therefore exogenous glycans can regulate gene expression in the host by a yet unknown mechanism (Angeloni et al., 2005). Milk oligosaccharides have been also shown to exhibit effects on growth, differentiation and apoptosis (Kuntz Sabine et al., 2008). *In vitro* stimulation of intestinal epithelial cell lines with milk oligosaccharides fractions induced growth inhibition by modulating epidermal growth factor signaling and gene expression of cell cycle regulators (Kuntz et al., 2009). This observation stands in contrast to the increased cell proliferation when total milk was analyzed on intestinal

cells (Ichiba et al., 1992), suggesting a possible control mechanism of intestinal cell growth by both milk oligosaccharides and total milk.

Systemic effects

A small amount of indigested milk oligosaccharides are absorbed by the infant's intestine by crossing epithelial layer via receptor-mediated transcytosi or paracellular transport (Gnoth et al., 2001). Detection of milk oligosaccharides in urine of breastfed, but not formula-fed infants suggests their existence in the systemic circulation (Obermeier et al., 1999; Rudloff et al., 1996). The appearance of milk oligosaccharides in circulation may alter certain protein-carbohydrate interactions. Putative carbohydrate binding partners of milk oligosaccharides include galectins, siglecs and selectins. The galectins recognize βGal-containing oligosacchiardes such as poly-N-acetyllactosamines found in the backbone structures of milk oligosaccharides. Galectins were shown to mediate cell adhesion, cell growth and regulate apoptosis (Perillo et al., 1998). The siglecs, sialic-acid-binding immunoglobulin-like lectins, recognize sialylated glycans and are involved in biological processes such as immune cell signaling (Varki and Angata, 2006). Selectins bind to specific fucosylated and sialylated oligosaccharides on their glycan ligands (Varki, 1994; Varki, 1997). Selectins are important during inflammation by mediating leukocyte adhesion, rolling and extravasation to the site of inflammation (Lasky, 1995; Varki, 1994). Milk oligosaccharides were shown to bear structures that resemble selectin ligands (Rudloff et al., 2002). Furthermore *in vitro* experiments confirmed the inhibitory action of sialylated milk oligosaccharides on rolling and adhesion of leukocytes (Bode et al., 2004). Therefore small amount of milk oligosaccharides in circulation might reduce intestinal inflammation by binding to selectin ligands and thus protect against inflammatory diseases (Bode, 2009) or mediate cell growth or apoptosis by binding galectin ligands.

The gastrointestinal immune system

The main function of the gastrointestinal tract is the absorption of nutrients, vitamins and water. To ensure optimal uptake the intestine is uniquely structured. Numerous complex foldings, the villi, enlarge the surface area up to 400 m^2. This is 200 times more than the total skin surface area and guaranties maximal absorption (Brandtzaeg, 2009). Shortly after birth the intestine is colonized by microorganisms. Approximately 10^{14} microorganisms, mainly bacteria, live in symbiosis with their host (Backhed et al., 2005). The bacteria profit from the energy rich environment whereas the host is delivered with vitamins and short-chain fatty acids. Moreover, commensal bacteria impede the colonization of pathogens and help the developing immune system. However, the constant exposure to potential pathogens and food antigens requires tightly regulated immune responses to discriminate self from non-self and danger from harmless signals. Therefore, the intestine comprises the largest and most complex immune system of the body (Mowat, 2003). Specialized cells of both the innate and adaptive immune system are located in the gut associated lymphoid tissues (GALT) orchestrating the balance between tolerance and inflammation to achieve protection and homeostasis.

Components of the mucosal immune system

Epithelial layer

The intestinal epithelium consists of a monolayer of columnar intestinal epithelial cells, the enterocytes. These cells are generated from stem cells in the crypt and migrate while maturing to the villus. Fully mature, they are equipped with enzymes for luminal and intracellular digestion and absorption. On the tip of the villi deceased cells are shed off in a cycle of 3-7 days in humans (Carethers, 1998). Specialized enterocytes, the goblet cells secrete mucus, high molecular weight glycoproteins, that overlay the intestinal layer providing a barrier for invading pathogens and neutralizing gastric acid (Specian and Oliver, 1991b). An additional epithelial cell lineage located to the crypts is the paneth cells. Through their release of antimicrobial peptides, they contribute immensely to the intestinal innate immunity by killing bacteria directly (Vaishnava et al., 2008). Intestinal cells that produce a wide range of hormones such as serotonin, motilin, cholecystokinin and vasoactive intestinal peptide are the so called endocrine cells (Flemstrom and Sjoblom, 2005). These cells are regulators of fluid and electrolyte secretion, motility and food intake by sensing luminal contents of nutrients, changes in osmolarity and acidity (Moran et al., 2008; Raybould, 2003). Interspersed among epithelial cells, specialized unconventional T

cells are found, the intraepithelial lymphocytes. These T cells are grouped into T cells containing either T cell receptor chains αβ or γδ. The T cells bear either co-receptor CD4 or CD8 where CD8 is found with αβ or αα chains (Cheroutre, 2004). Their function is as complex as their phenotype varying from cytolytic or immune regulatory actions to tissue repair (Fig. 6).

Peyer's patches

The Peyer's patches are lymphoid structures that are located to the submucosa along the small intestine. They are the inductive sites of the mucosal immune system containing B cell areas and adjacent T cell areas. These lymphoid areas are separated from the lumen by a single cell layer, the follicle-associated epithelium, and a diffuse area containing B cells, T cells and dendritic cells, the so called subepithelial dome region. Features of the follicle-associated epithelium such as low expression levels of membrane-associated digestive hydrolases, small amounts of mucus production and antimicrobial peptides, enhance their contact with foreign antigens and pathogens. Specialized transporter cells, the microfold cells, deliver antigens from the lumen to the subepithelial dome where dendritic cells capture the antigens and induce immunity (Didierlaurent et al., 2002; Mowat, 2003; Neutra et al., 2001) (Fig. 5).

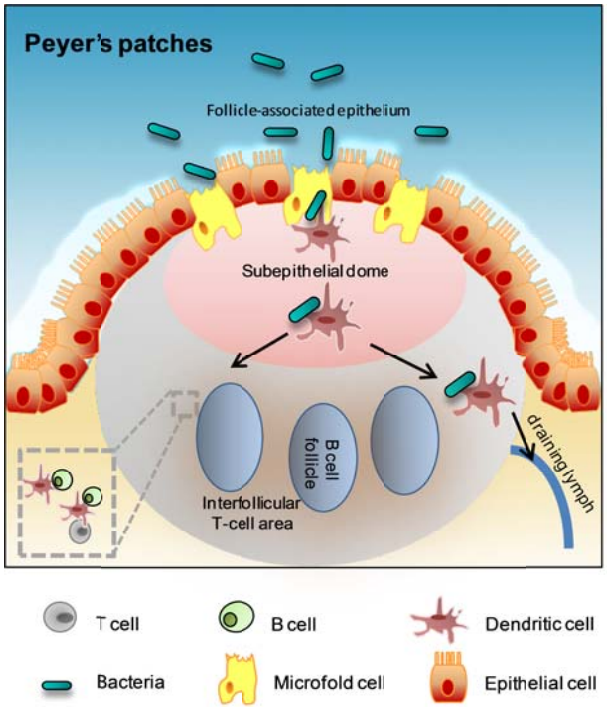


Figure 5. The Peyer's patches.

Antigens enter through the microfold cells in the follicle-associated epithelium and after transfer to local dendritic cells in the subepithelial dome are presented directly to T cells in the Peyer's patches. Alternatively, dendritic cells containing bacteria gain access to draining lymph with subsequent T cell recognition in the mesenteric lymph nodes. (modified from (Macpherson and Harris, 2004; Mowat, 2003).

Isolated lymphoid follicles and cryptopatches

The isolated lymphoid follicles are aggregations of lymphoid structures in the intestinal wall. They resemble Peyer's patches and are additional inductive sites for mucosal T and B cells. Cryptopatches are clusters of lymphoid cells in the basal lamina propria (Brandtzaeg and Pabst, 2004). Their occurrence is species specific and believed to represent precursors of isolated lymphoid follicles rather than being sites of extrathymic intraepithelial lymphocyte development as previously thought (Brandtzaeg et al., 2008; Onai et al., 2002).

Mesenteric lymph nodes

The mesenteric lymph nodes are the largest lymph nodes of the body. They are known to be the main site for oral tolerance induction (Mowat et al., 2004). Food antigens from the intestinal lumen are picked up by dendritic cells from the intestinal epithelium and from the subepithelial dome in the Peyer's patches, migrate to the mesenteric lymph nodes where they are displayed to T cells for tolerance induction (Strobel and Mowat, 2006; Worbs et al., 2006). Despite this effect, mesenteric lymph nodes are the key players to preserve systemic ignorance to commensal bacteria. The mesenteric lymph nodes build a "firewall" compartmentalizing the mucosal from the systemic immune system (Macpherson and Uhr, 2004). Therefore, when bacteria are transported via dendritic cells to the mesenteric lymph nodes they are quickly eliminated by macrophages, preventing their systemic penetration (Macpherson and Smith, 2006).

Lamina propria

The lamina propria is a thin layer of connective tissue underneath the epithelial layer. It is known to be the effector site of the mucosal immune system (Brandtzaeg and Pabst, 2004). More than 80% of all plasma cells in the body are located to the lamina propria producing IgA amounts which exceed the total production of all other immunoglobulin isotypes combined (Fagarasan and Honjo, 2003; Macpherson and Harris, 2004). Dimeric IgA are transported through the epithelial layer to the intestinal lumen via receptor coupled vesicular transport (Brandtzaeg and Prydz, 1984). In the lumen, IgA opsonize bacteria and prevent their attachment to the epithelial layer and their invasion. T cells residing in the lamina propria are of an effector or memory phenotype. After priming in the mesenteric lymph nodes by antigen-loaded dendritic cells T cells

migrate home to the intestine. This homing is performed by the expression of α4ß7 integrin and the chemokine receptor CCR9 (van Wijk and Cheroutre, 2009). Along with effector T cells, a distinct cell population, the regulatory T cells, reside in the lamina propria, regulating pro and anti-inflammatory immune responses (Barnes and Powrie, 2009). Professional antigen-presenting cells, the dendritic cells, are frequently found in the lamina propria. They can sample antigens directly from the intestinal lumen by extending their dendrites across the epithelial layer (Rescigno et al., 2001)and induce IgA-class switching or differentiation *in situ* (Fagarasan and Honjo, 2003) (Fig. 6).

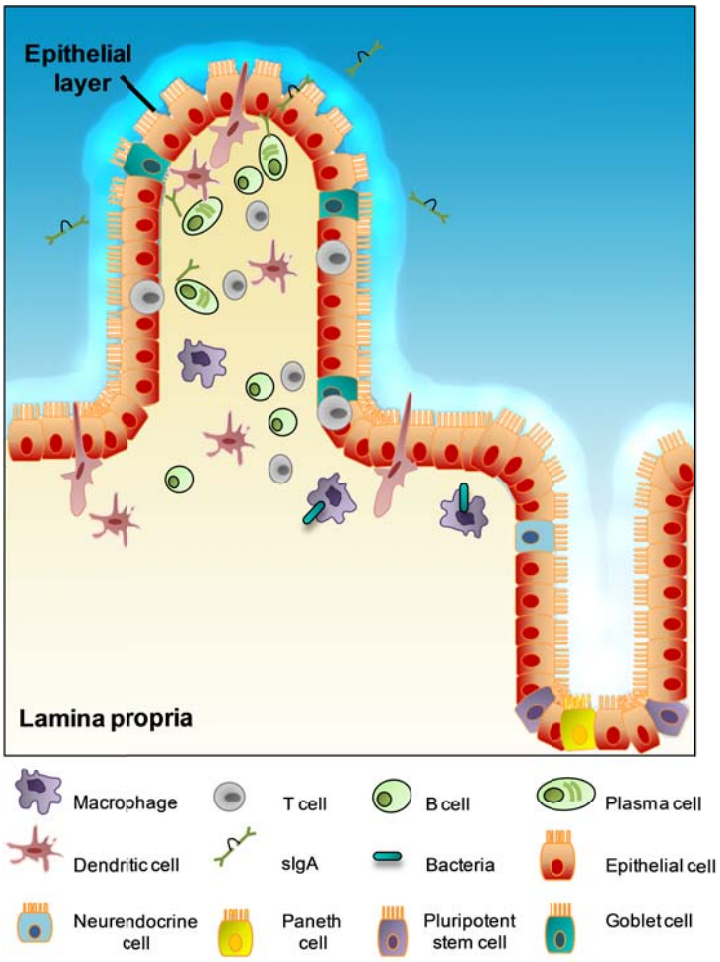


Figure 6. Intestinal epithelial cell layer and lamina propria.

The intestinal epithelial layer is composed of absorptive epithelial cells (enterocytes), antimicrobial peptide releasing paneth cells, dividing pluripotent stem cells, neuroendocrine cells and mucin-releasing goblet cells. The lamina propria is composed of macrophages that directly kill bacteria entering through the epithelial layer, plasma cells that secrete IgA, dendritic cells that can take up antigens and present them directly to T and B cells and also effector and T effector cells.

First line of defense

The constant exposure of the gastrointestinal tract to foreign antigens demands specific physical adaption to prevent over reactions of the immune system. This is provided by the first line of defense which consists of extrinsic (outside the epithelial monolayer) and intrinsic (inside the epithelial monolayer) barriers between the epithelial cells and the environment.

Gastric acid is part of the extrinsic chemical barrier. It lowers the pH in the gastrointestinal tract leading to a decrease in bacterial activity, an increase in bacterial cell wall lysis and an increase in detoxification of toxins (Giannella et al., 1972). Goblet cells in contrast provide the extrinsic physical barrier. They secrete high molecular weight glycoproteins, the mucins, that form a hydrated viscous layer (Maury et al., 1995). This mucosal blanket serves as a size selective diffusion barrier allowing small particles to pass while trapping bacteria inside (Frey et al., 1996). Bacteria are trapped in the mucus layer by binding to carbohydrate moieties of mucins that mimick epithelial cell membrane glycans used for pathogen adherence (Chadee et al., 1987) or by binding membrane component of pathogens such as type 1 pili of Escherichia coli (Sajjan and Forstner, 1990). The mucus containing bacteria is sequentially washed away through the peristaltic movements of the intestine to prevent their overgrowth and translocation (Pitman and Blumberg, 2000; Specian and Oliver, 1991a).

The intrinsic physical barrier, the epithelial cell monolayer is maintained by two structural components: the brush border and the tight junctions. The brush border is formed by finger-like microvillar extensions on intestinal epithelial cell protecting their apical side. Intercellular tight junctions connect adjacent cells and regulate paracellular permeability. They are linked to the brush border through an actin filament network. Together they ensure strong membrane integrity and inhibit bacterial translocation (Artis, 2008; Shen and Turner, 2006; Turner, 2009; Wang and Andersson, 1994). The epithelial monolayer is constantly replaced by dividing cells in the crypt that move up towards the villi where they undergo apoptosis. This constant replacement eliminates infected or damaged cells rapidly (Potten et al., 1997). In the case of a chemical or toxic injury, the renewal and migration of intestinal epithelial cells toward injured sites is enhanced by intestinal trefoil factor, a peptide secreted into the mucous layer by goblet cells (Pitman and Blumberg, 2000; Podolsky, 1999). This rapid adaptation provides innate resistance against epithelial insults by maintaining cellular integrity.

Another biochemical barrier is produced by the paneth cells located at the base of small intestinal crypts. They are specialized epithelial cells that store granules containing antimicrobial

proteins, such as lysozymes, secretory phospholipase A_2 and defensins (Ganz, 2003; Harwig et al., 1995; Selsted and Ouellette, 2005). Upon sensing bacterial signals, paneth cells release their microbicidal granules that kill bacteria through compromising the integrity of the bacterial cell wall (Ayabe et al., 2000; Ouellette, 1999). The lack of paneth cells leads to increased bacterial translocation into host tissue, emphasizing the importance of antimicrobial proteins in the retention of bacteria to the intestinal lumen (Vaishnava et al., 2008).

Indispensible for protection from invasive bacteria is the production of secretory IgA (Macpherson et al., 2001). The generation of IgA depends on the help of dendritic cells that sample bacteria from the subepithelial dome in the Peyer's patches or directly from the intestinal lumen through lamina propria dendritic cells. Dendritic cells containing engulfed bacteria induce B cells to differentiate into plasma cells producing IgA specific for intestinal bacteria (Macpherson and Uhr, 2004). The transcytosed IgA across the epithelial layer binds to bacteria in the lumen preventing their association with the intestinal epithelial surface (Mostov, 1994); Machperson 2007) (Fig. 7).

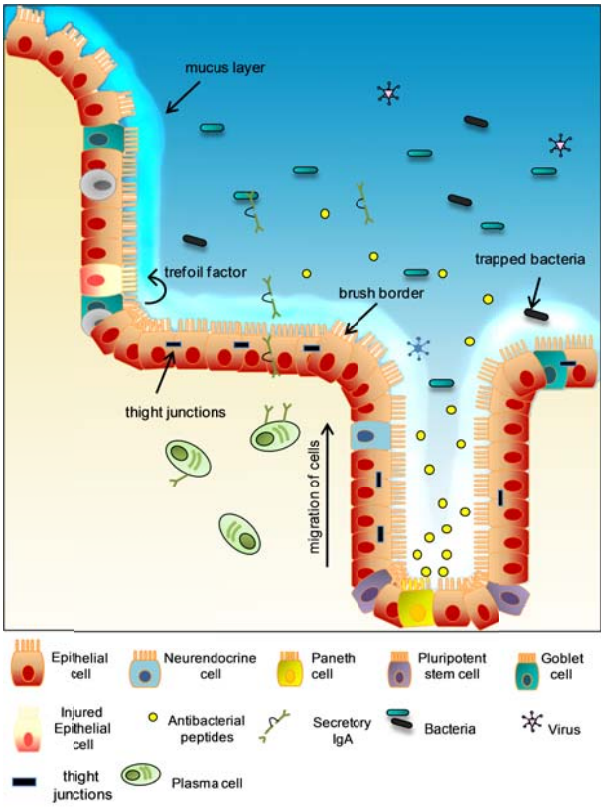


Figure 7. First line of defence in the gastrointestinal tract.

Mechanisms by which invasion of potential pathogens is prevented include mucus layer, brush border membrane, tight juctions, cell migration/renewal, antimicrobial peptides and secretory IgA.

Intestinal microbiota

Shortly after birth the gastrointestinal tract is colonized with up to 100 trillion (10^{14}) microbes (Savage, 1977). These microbes include all three domains of life, bacteria archaea and eukarya. The distal gut, the colon, is the most densely populated part of the intestine. It is recorded to be the densest habitat in nature with approximately 10^{11}-10^{12} cells/ml. (Whitman et al., 1998). Despite this cellular density, the diversity at the division level is low compared to other ecosystems (Hugenholtz et al., 1998). More than 90% of the gut microbiota are gram-negative anaerobes and belong to only two of the 70 known bacterial divisions, the *Firmicutes* and the *Bacteroidetes*. The remaining 10% of the gut microbiota belong to *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, *Spirochaetes* and *Cyanobacteria* (Backhed et al., 2005; Eckburg et al., 2005; Ley et al., 2008). In contrast to their low diversity at the division level, intestinal microbiota is rich at the species and strain level, with an estimate of 500-1000 different species and over 7000 strains (Dethlefsen et al., 2007; Ley et al., 2006a). The indigenous microbiota, the commensals, coevolved with their hosts and became indispensible for host health. However under specific conditions, such as loss of tolerance to bacteria or opportunistic infections, they overcome protective host properties and exert pathologic effects (Abt and Artis, 2009) (Fig. 8).

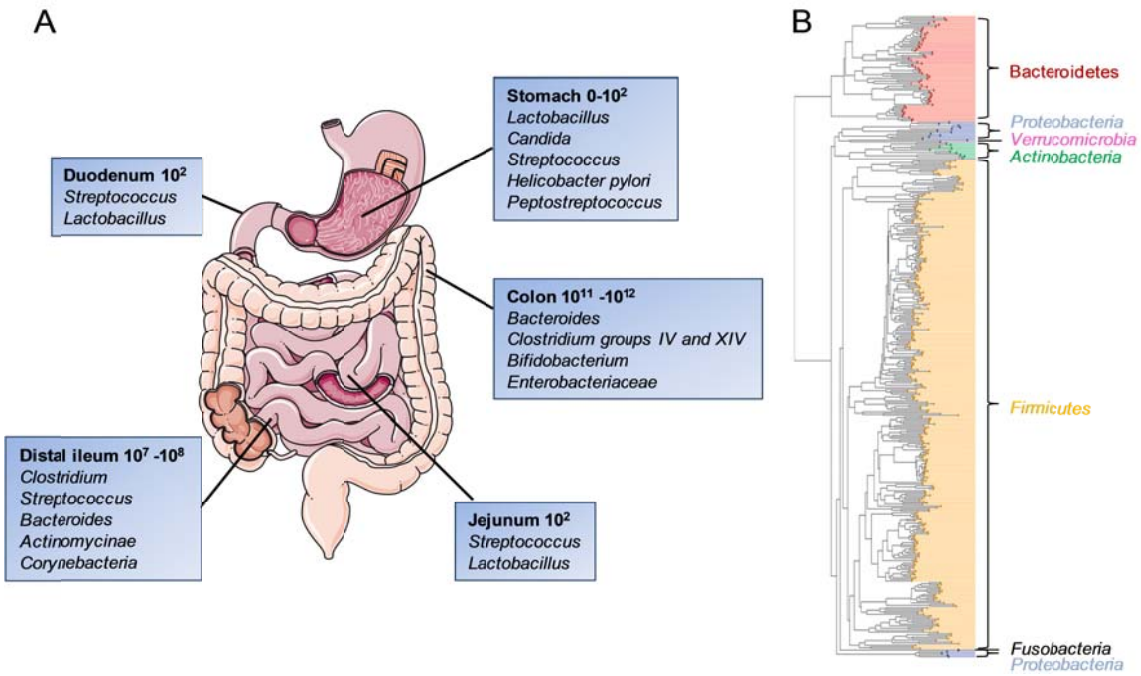


Figure 8. Intestinal microbiota

(A) Compostion and luminal concentration of dominant microbial species in the stomach, duodenum, jejunum, distal ileum and colon. (B) Phylogenetic analysis of 16S rDNA sequences amplified from feces of

the human colon. *Firmicutes* and *Bacteroidetes* are the dominant phyla whereas *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* are less abundant (modified from Sartor, 2008; Eckburg et al., 2005)

Contributions of the microbiota

The generation and housing of germ-free animals revealed important information about the effects that microbes exhibit on host physiology and pathology (Falk et al., 1998). Analysis of germ-free animals compared to conventionally raised animals showed structural and functional differences such as enlarged cecum, diffuse and underdeveloped gut associated lymphoid tissue and angiogenesis (Stappenbeck et al., 2002), altered gut epithelial cell turnover (Savage et al., 1981) and increased caloric intake (Wostmann, 1981).

A major metabolic function of the intestinal microbiota lies in their capability to hydrolyse complex polysaccharide structures derived from a plant-rich diet (Savage, 1986). The limited repertoire of glycosylhydrolases in the mammalian genome is insufficient for optimal energy harvest. Microbes provide the needed saccharolytic enzymes digesting otherwise indigestible polysaccharides. Thus the microbiota ensures optimal energy uptake and allows rapid adaption to dietary changes (Duerkop et al., 2009; Hooper et al., 2002). Alteration in the composition of microbial divisions can lead to metabolic disorders such as obesity and diabetes. In obese individuals, for example, the proportion of *Firmicutes* to *Bacteroidetes* is shifted to a higher number of *Firmicutes* whereas in lean individuals *Bacteroidetes* are dominant (Ley et al., 2006b). End products of bacterial fermentation, the short chain fatty acids are the main source of energy for the colonic epithelial cells (Bergman, 1990). However not only do they deliver energy, they also promote proliferation and differentiation of intestinal crypt cells (McCullough et al., 1998). Furthermore, colonization studies with ex-germfree animals revealed an impact of the microbiota on the immune system. Germfree housed animals have extensive defects in the development of the gut-associated lymphoid tissues with fewer and smaller Peyer's Patches and mesenteric lymph nodes, impaired development of isolated lymphoid follicles and lower antibody production (Bouskra et al., 2008; Macpherson and Harris, 2004). Intestinal microbes additionally provide signals for the development of key lymphocyte populations. They for example induce IgA class switching in intestinal B lymphocytes (He et al., 2007). Upon interaction with antigens presented by dendritic cells, $CD4^+$ T cells can differentiate into a variety of effector subsets, namely Th1, Th2, Th17 and T regulatory cells in order to protect the host against different classes of pathogens (Zhou et al., 2009). Th1 cells are involved in cellular immunity against intracellular microorganisms, whereas Th2 cells helps clearing extracellular bacteria, fungi and helminthes

(Sallusto and Lanzavecchia, 2009). Th17 are especially important at musosal sites for the elimination of extracellular bacteria and fungi (Weaver et al., 2007). Regulatory T cells are crucial for immune homeostasis as they suppress immune activation by releasing anti-inflammatory cytokines (Vignali et al., 2008). These T helper subsets were shown to be influenced by the composition of the microbiota. Thus, the microbiota regulated the balance between Th17 and Treg cells in the lamina propria (Bettelli et al., 2007; Ivanov et al., 2008). Furthermore the microbiota has an impact on systemic immune response by influencing the Th1 and Th2 effector cells. Bacterial-derived polysaccharide A from the commensal *Bacteroides fragilis* was shown to affect systemic Th1 response by inducing $CD4^+$ T cells proliferation and Th1 cytokine production (Mazmanian et al., 2005).

Probiotics

Probiotics were first described as nonpathogenic living microorganisms, which exhibit beneficial effects on host health and contribute to disease prevention (Lilly and Stillwell, 1965). Their properties are defined through their ability to resist gastric juice, pass through the gastrointestinal tract and proliferate in the gut in the presence of bile and anaerobic conditions. These criteria are mainly matched by *Lactobacillus*, *Bifidobacterium* and *Saccharomyces* (Holzapfel et al., 2001) although not excluding microorganisms from other genera such as *E.coli Nissle 1917*. Through improved techniques in microbial typisation and increasing evidence of the close interplay between the host genome and its microenvironment, probiotics have gained more attention in current research. The accumulated knowledge about the mechanisms, by which probiotics promote host health, makes their supplementation a possible treatment for microbiota associated diseases such as inflammatory bowel disease (Reiff and Kelly, 2010).

Functions of probiotics

Probiotics contribute to host homeostasis by different mechanisms. The following three approaches show how probiotics exhibit beneficial effect (Fig. 9).

The first approach involves the production of short-chain fatty acids and antibacterial peptides, the so called bacteriocins. The short-chain fatty acids produced by certain probiotics during the anaerobic metabolism of carbohydrates create a low pH environment that limits bacterial growth (Louis et al., 2007). Bacteriocins are ribosomally synthesized antimicrobial peptides produced by one bacterium that kill other bacteria but simultaneously are immune to their own bacteriocins

(Riley and Wertz, 2003). Bacteriocins eliminate potential pathogens by pore formation in the cytoplasmic membrane of sensitive bacteria (Cotter et al., 2005; Vesterlund et al., 2004) Together with competitive inhibition of pathogen and toxin adherence to intestinal epithelial cells or mucus, probiotics act as a barrier against a potential pathogenic microbial environment (Candela et al., 2005; Collado et al., 2007).

The second approach involves the regulatory effect of probiotics on immune function. One mechanism involves the enhancement of host adaptive immunity. Probiotics have the potential to prime intestinal dendritic cells to selectively activate B lymphocytes to produce IgA, that once transported into the lumen reduces bacterial penetration through the intestinal layer (Macpherson and Uhr, 2004). Intestinal dendritic cells are important in maintenance of tolerance towards commensal bacteria and the generation of an immune response against pathogens. They can sense the local microbe rich environment and use the signals for induction of an appropriate immune response (Coombes and Powrie, 2008). For example when recognizing bacteria of a probiotic or commensal nature, low-level immune response is induced characterized by low expression of costimulatory molecules or inflammatory cytokines. In contrast, when dendritic cells sense pathogens they are primed to mount high Th1 immune response (Braat et al., 2004; Veckman et al., 2004). Additional probiotics prime dendritic cells to produce anti-inflammatory cytokines such as IL-10 to down modulate Th1 response (Drakes et al., 2004). Thus, probiotics and commensal bacteria balance the immune system between pro- and anti-inflammatory mucosal immune response leading to mucosal immune homeostasis.

The third approach involves the regulation of intestinal epithelial cell function by probiotics. A functional intestinal barrier is crucial for host protection. Probiotic *E. coli Nissle 1917* for example was shown to upregulate tight junction proteins after enteropathogenic *E. coli* induced injury, thereby initiating tissue repair (Zyrek et al., 2007). The probiotics *Streptococcus thermophilus* and *lactobacillus acidophilus* were reported to protect intestinal barrier function by prevention of cytokine-induced increases of intestinal epithelial paracellular permeability (Resta-Lenert and Barrett, 2006). Epithelial cells are also directly influenced by probiotics by an enhanced production of cytoprotective heat shock proteins and antibacterial peptides such as β-defensins (Tao et al., 2008; Wehkamp et al., 2004).

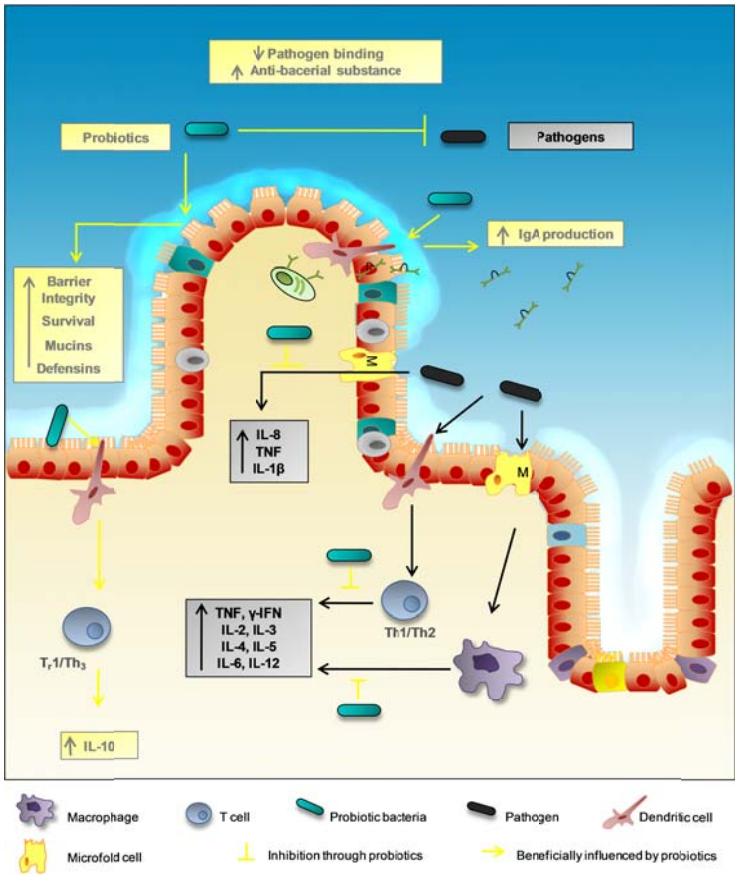


Figure 9: Regulation of host homeostasis by probiotics.

Probiotics positively influence intestinal epithelial cell function and immunity by increasing barrier integrity, survival, mucins, defensins and IgA production and by priming dendritic cells to induce anti-inflammatory IL-10 (yellow arrows). Probiotics also block effects of intestinal pathogenic bacteria (yellow blocking sign) by inhibiting colonization of pathogenic bacteria and by blocking pro-inflammatory cytokines (Modified from Vanderpool et al. 2008).

*Toll*erance or *NOD*

Innate immunity response relies on recognition of conserved microbial structures the pathogen-associated molecular patterns. These structures are recognized through pattern recognition receptors expressed on various immune cells and on epithelial cells adjoining microbe rich environments (Medzhitov and Janeway, 2000). The patterns recognized by these receptors include nucleic acids, surface glycoproteins and lipoproteins and bacterial cell wall components such as peptidoglycans and LPS. They are exclusively produced by microbes and not by the host itself allowing the innate immune system to distinguish between “self” and “microbial non-self” (Janeway, 1992; Medzhitov and Janeway, 1997).

A diverse set of pattern recognition receptors exists that can be categorized into secreted, cytosolic and transmembrane receptors. Secreted pattern recognition receptors such as. collectins and pentraxins, activate the pathways of the complement system and opsonize microbes for phagocytosis by immune cells (Bottazzi et al., 2010). Cytosolic pattern recognition receptors include the retinoic acid inducible gene I like receptors and the nucleotide-binding domain and leucine-rich repeat-containing receptors (Inohara et al., 2005; Meylan and Tschopp, 2006). The nucleotide-binding oligomerization domain (NOD) receptors sense bacterial molecules produced during the degradation of the cell wall polymer peptidoglycan such as muramyl dipeptide (Fritz et al., 2006). Transmembrane pattern recognition receptors include two families, the Toll-like receptors (TLR) and C-type lectin receptor (Rakoff-Nahoum et al., 2004; Weis et al., 1998). The TLRs are the best characterized transmembrane pattern recognition receptors. They are expressed either on the plasma membrane or in endosomal/lysosomal organelles.

Intestinal epithelial cells express pattern recognition receptors and respond to commensal bacteria. To limit inappropriate immune activation to commensal bacteria at the mucosal surface, pattern-recognition receptors exhibit restricted expression and are well regionally and functionally compartmentalized (Abreu, 2010) (Fig. 10). For example, the expression levels of TLR4 and TLR2 and the corresponding co-receptors CD14 and MD-2 are kept low in the intestinal epithelial cells and are therefore relatively unresponsive to their ligands (Abreu et al., 2001; Melmed et al., 2003). Although TLR4 expression and signaling is thought to occur at the plasma membrane, TLR4 was also found in the Golgi apparatus (Hornef et al., 2002). Co-localization experiments confirmed an interaction of internalized LPS with TLR4. This internalization of LPS was shown to be required for TLR4 signaling in the intestine (Hornef et al., 2003). This compartmentalization points out another precaution in preventing nonspecific activation of TLR. Another mechanism to compartmentalize TLRs is by their expression at the basolateral surface of epithelial cells as seen by TLR5, the recognition receptor for flagellin. Thus stimulation of TLR5 requires trans-epithelial transport of bacterial flagellin or leakage of epithelial tight junctions. TLR localization therefore ensures activation only by bacterial invasion and prevents hyperresponsiveness at the mucosal surface (Gewirtz et al., 2001). Not only receptor location but also their function can be polarized. TLR9, the receptor for unmethylated CpG DNA, is expressed on both the apical and basolateral side of intestinal epithelial cells. TLR9 engagement on the apical side results in an inhibition of the NF-κB pathway whereas basolateral engagement activates the pathway and induces a pro-inflammatory response (Lee et al., 2006).

An additional control of TLR activation is provided by negative regulators of Toll-like receptor-mediated signaling. Toll-interacting protein inhibits IL-1 and NF-κB activation during TLR2 and TLR4 engagement though suppression of kinase activity of IRAK (Zhang and Ghosh, 2002). The single immunoglobulin IL-1R-related molecule functions as a decoy receptor interfering with the recruitment of downstream adaptors to the TLR complex during TLR4 and TLR9 signaling (Wald et al., 2003). The peroxisome proliferator activated receptor-γ also negatively regulates NF-κB activation in a TLR4 dependant manner (Dubuquoy et al., 2003). The prolonged exposure of TLR ligands or of microbiota extracts upregulate the expression of both inhibitors Toll-interacting protein and peroxisome proliferator-activated receptor-γ (Kelly, 2004; Melmed et al., 2003). Thus, they down-regulate TLR activity in response to the continual exposure of intestinal epithelial cells to commensal bacteria enforcing tolerance. In contrast to pathogenic bacteria, commensals are also able to directly down-regulate pro-inflammatory responses by mechanisms such as blocking the NF-κB pathway through inhibition of I-κB ubiquitination (Kelly, 2004; Neish, 2000).

Apart from TLR mediated upregulation of inflammatory cytokines via the NF-κB pathway, TLRs are also involved in the induction of IgA and antimicrobial peptides, and repair of epithelial cell injury through the induction of proliferation and tight junctions. A beneficial role in maintaining intestinal homeostasis was shown by deletion of TLRs or the adaptor protein MyD88 as the corresponding knock-out mice are not protected from intestinal inflammation (Abreu, 2010).

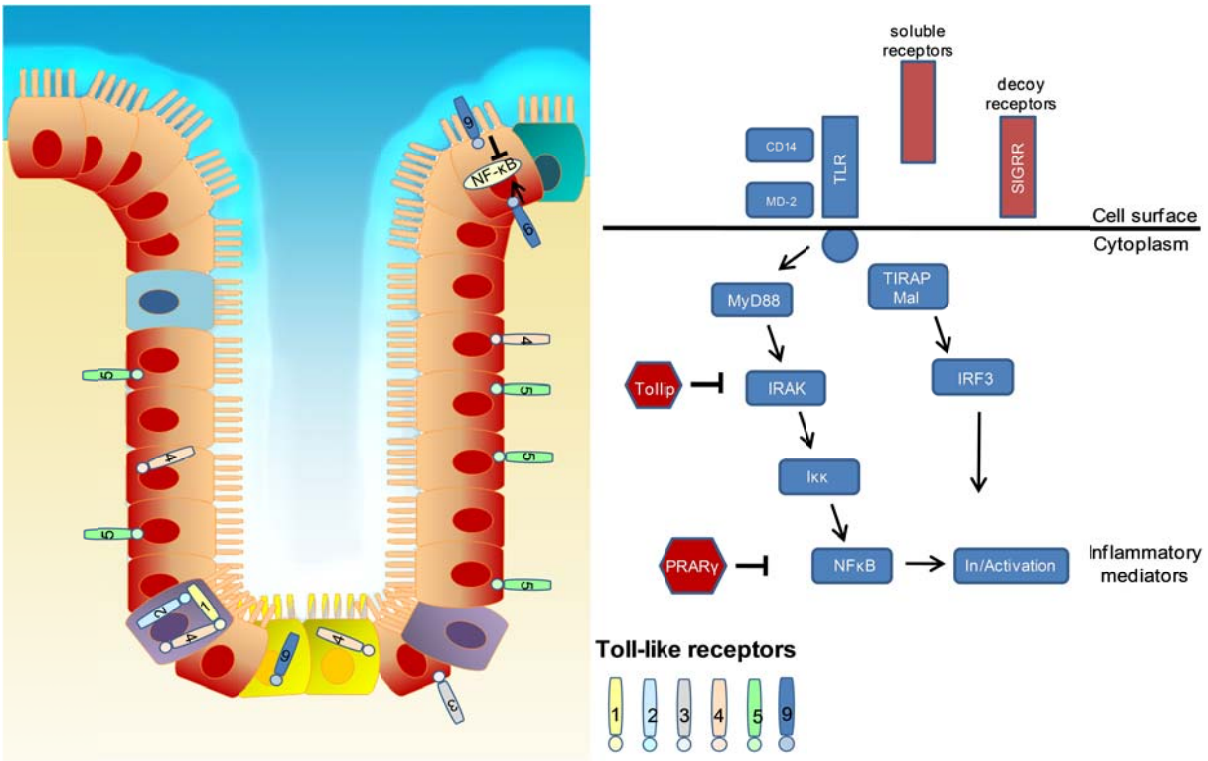


Figure 10: Toll-like receptor localization and signaling in the intestine.

TLRs in the intestine are localized prevalently on the basolateral side of intestinal epithelial cells or intracellularly (left figure). The activation (Wen et al.) and inhibition (red) of the TLR signaling cascade are depicted (right figure). Tollip: Toll interacting protein; PRARγ: peroxisome proliferator-activated receptor; TLR: Toll-like receptor; MyD88: myeloid differentiation primary response gene (88); IRAK: IL-1 receptor-associated kinase; NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells; Ικκ : IκB kinase; TIRAP: toll-interleukin 1 receptor domain containing adaptor protein; IRF3: IFN-regulatory factor 3; SIGGR: single immunoglobulin IL-1R-related molecule.

When homeostasis fails: Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract. The two main forms of IBD are Crohn's disease and ulcerative colitis. Ulcerative colitis starts in the rectum and spreads along the colon. Inflammation is characterized through superficial ulcerations of the mucosa with infiltrating lymphocytes and granulocytes and loss of goblet cells. Crohn's disease on the other hand affects the whole gastrointestinal tract and is characterized by transmural inflammation with large ulcerations and occasional granuloma (Bouma and Strober, 2003). Despite the high prevalence range of 10-200 per 100'000 individuals in North

America and Europe, the exact cause of the disease has not yet been elucidated. However, there is a general agreement that a complex interplay between genetic, environmental and immunological factors is involved in disease initiation and progression (Bouma and Strober, 2003; Sartor, 2008; Sokol et al., 2008).

It is believed that innate immune response to commensal bacteria plays an important role in the initiation of IBD and in the development of pathological adaptive immune responses. Hence, an imbalance between pathogenic and protective microorganisms or a loss of gut bacteria that promote tolerance and regulatory T cell polarization could lead to an aberrant inflammatory response. As a result hyperresponsive Th1 or Th2 cells develop seen in Crohn's disease and ulcerative colitis (Podolsky, 2002).

Genetic factors have been investigated by genome-wide searches for IBD susceptibility gene loci. The first one identified was NOD2 followed by others such as IBD5, IL23R and ATG16L1 (Duerr, 2006; Hampe, 2007; Hugot et al., 2001; Ogura, 2001; Silverberg, 2007). Interestingly all these genes were implicated in innate immunity, phagocytosis or autophagy. NOD2 mutation in patients with Crohn's disease showed impaired NF-κB activation, a key transcription factor involved in inflammation and immune processes. Moreover Crohn's disease patients have reduced expression of the antimicrobial peptide β-defensin, leading to enhanced bacterial translocation (Wehkamp et al., 2005). Additional polymorphisms and altered expression patterns of TLRs were detected in patients with IBD (Cario and Podolsky, 2000; Franchimont, 2004). This illustrates that the homeostasis between microbes and in particular the commensal flora and host immune response at the mucosal site is crucial particularly in preventing the pathogenesis of IBD. This hypothesis was supported by the beneficial effects of antibiotic treatment in some patients during active IBD and the amelioration of the disease after probiotics administration (Sartor, 2004). Furthermore, animal models of spontaneous colitis are entirely dependent on the presence of the microbiota as they remain healthy in a germfree environment (Horwitz, 2007).

Unfortunately to date there is no cure for IBD. However, new insights in the pathogenesis of IBD revealed potential therapeutic approaches, of which some are now being tested in clinical trials. These include therapeutics in T cell polarization/differentiation, modulation of inflammatory cytokines/pathways such as blockage of the inflammatory cytokine TNF-α, inhibition of leukocyte infiltration, intestinal barrier repair and probiotics (Korzenik and Podolsky, 2006). Moreover, selected oligosaccharides are promising new candidates in the treatment of IBD. They function as prebiotics that alter the intestinal microbial composition by stimulating the growth of protective

bacteria. They enhance colonization resistance of disease-inducing bacteria and contribute to a reduction in colitis (Gibson et al., 2004; Looijer-Van Langen and Dieleman, 2009).

References

Abrams, S.A., I.J. Griffin, K.M. Hawthorne, L. Liang, S.K. Gunn, G. Darlington, and K.J. Ellis. 2005. A combination of prebiotic short- and long-chain inulin-type fructans enhances calcium absorption and bone mineralization in young adolescents. *Am J Clin Nutr* 82:471-476.

Abreu, M.T. 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol* 10:131-144.

Abreu, M.T., P. Vora, E. Faure, L.S. Thomas, E.T. Arnold, and M. Arditi. 2001. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *J Immunol* 167:1609-1616.

Abt, M.C., and D. Artis. 2009. The intestinal microbiota in health and disease: the influence of microbial products on immune cell homeostasis. *Curr Opin Gastroenterol.* 25:496-502.

Anderson, J.W., B.M. Johnstone, and D.T. Remley. 1999. Breast-feeding and cognitive development: a meta-analysis. *Am J Clin Nutr* 70:525-535.

Andersson, B., O.L.A. Porras, L. Hanson, T. Lagergard, and C. Svanborg-Eden. 1986. Inhibition of attachment of Streptococcus pneumoniae and Haemophilus influenzae by human milk and receptor oligosaccharides. *J Infect Dis* 153:232-237.

Angeloni, S., J.L. Ridet, N. Kusy, H. Gao, F. Crevoisier, S. Guinchard, S. Kochhar, H. Sigrist, and N. Sprenger. 2005. Glycoprofiling with micro-arrays of glycoconjugates and lectins. *Glycobiology* 15:31-41.

Artis, D. 2008. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat Rev Immunol* 8:411-420.

Ayabe, T., D.P. Satchell, C.L. Wilson, W.C. Parks, M.E. Selsted, and A.J. Ouellette. 2000. Secretion of microbicidal α-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol* 1:113-118.

Backhed, F., R.E. Ley, J.L. Sonnenburg, D.A. Peterson, and J.I. Gordon. 2005. Host-Bacterial Mutualism in the Human Intestine. *Science* 307:1915-1920.

Barnes, M.J., and F. Powrie. 2009. Regulatory T Cells Reinforce Intestinal Homeostasis. *Immunity* 31:401-411.

Bergman, E.N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70:567-590.

Bettelli, E., M. Oukka, and V.K. Kuchroo. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol.* 8:345-350.

Bode, L. 2009. Human milk oligosaccharides: prebiotics and beyond. *Nutr Rev.* 67:S183-S191.

Bode, L., S. Rudloff, C. Kunz, S. Strobel, and N. Klein. 2004. Human milk oligosaccharides reduce platelet-neutrophil complex formation leading to a decrease in neutrophil β 2 integrin expression. *J Leukoc Biol* 76:820-826.

Boehm, G., and B. Stahl. 2007. Oligosaccharides from Milk. *J Nutr.* 137:847S-849.

Bottazzi, B., A. Doni, C. Garlanda, and A. Mantovani. 2010. An Integrated View of Humoral Innate Immunity: Pentraxins as a Paradigm. *Annu Rev Immunol.* 28:157-183.

Bouchara, J.P., M. Sanchez, A. Chevailler, A. Marot-Leblond, J.C. Lissitzky, G. Tronchin, and D. Chabasse. 1997. Sialic aciddependent recognition of laminin and fibrinogen by Aspergillus fumigatus conidia. *Infect Immun.* 65:2717–2724.

Bouma, G., and W. Strober. 2003. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 3:521-533.

Bouskra, D., C. Brézillon, M. Bérard, C. Werts, R. Varona, I.G. Boneca, and G. Eberl. 2008. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature* 456:507-510.

Braat, H., E. Jong, J.H. Brande, M. Kapsenberg, M. Peppelenbosch, E.F. Tol, and S.H. Deventer. 2004. Dichotomy between Lactobacillus rhamnosus and Klebsiella pneumoniae on dendritic cell phenotype and function. *J Mol Med.* 82:197-205.

Brandtzaeg, P. 2003. Mucosal immunity: integration between mother and the breast-fed infant. *Vaccine* 21:3382-3388.

Brandtzaeg, P. 2009. Mucosal immunity: induction, dissemination, and effector functions. *Scand J Immunol.* 70:505-515.

Brandtzaeg, P. 2010. The mucosal immune system and its integration with the mammary glands. *J Pediatr.* 156:S8-S15.

Brandtzaeg, P., H. Kiyono, R. Pabst, and M.W. Russell. 2008. Terminology: nomenclature of mucosa-associated lymphoid tissue. *Mucosal Immunol* 1:31-37.

Brandtzaeg, P., and R. Pabst. 2004. Let's go mucosal: communication on slippery ground. *Trends Immunol.* 25:570-577.

Brandtzaeg, P., and H. Prydz. 1984. Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins. *Nature* 311:71-73.

Brinkman-Van der Linden, E.C.M., E.R. Sjoberg, L.R. Juneja, P.R. Crocker, N. Varki, and A. Varki. 2000. Loss of N-Glycolylneuraminic Acid in Human Evolution. *J Biol Chem.* 275:8633-8640.

Candela, M., G. Seibold, B. Vitali, S. Lachenmaier, B.J. Eikmanns, and P. Brigidi. 2005. Real-time PCR quantification of bacterial adhesion to Caco-2 cells: Competition between bifidobacteria and enteropathogens. *Res Microbiol.* 156:887-895.

Carethers, J.M. 1998. Cell checkpoints and enterocyte differentiation: a recipe for sequential stages Focus on "Caco-2 intestinal cell differentiation is associated with G1 arrest and suppression of CDK2 and CDK4". *Am J Physiol.* 275:C1191-1192.

Cario, E., and D.K. Podolsky. 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun.* 68:7010-7017.

Chadee, K., W.A. Petri, D.J. Innes, and J.I. Ravdin. 1987. Rat and human colonic mucins bind to and inhibit adherence lectin of Entamoeba histolytica. *J Clin Invest.* 80:1245-1254.

Cheroutre, H. 2004. Starting at the beginning: new perspectives on the biology of mucosal T cells. *Annu Rev Immunol.* 22:217-246.

Collado, M.C., J. Meriluoto, and S. Salminen. 2007. Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. *Lett Appl Microbiol.* 45:454-460.

Coombes, J.L., and F. Powrie. 2008. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol* 8:435-446.

Coppa, G.V., S. Bruni, L. Zampini, T. Galeazzi, B. Facinelli, R. Capretti, A. Carlucci, and O. Gabrielli. 2003. Oligosaccharides of human milk inhibit the adhesion of Listeria monocytogenes to Caco-2 cells. *Ital J Pediatr* 29:61-68.

Cotter, P.D., C. Hill, and R.P. Ross. 2005. Bacteriocins: developing innate immunity for food. *Nat Rev Micro* 3:777-788.

Cravioto, A., R.E. Reyes, F. Trujillo, F. Uribe, A. Navarro, J.M. De la Roca, J.M. Hernandez, G. Perez, and V. Vazquez. 1990. Risk of diarrhea during the first year of life associated with initial and subsequent colonization by specific enteropathogens. *Am J Epidemiol.* 131:886-904.

Dethlefsen, L., M. McFall-Ngai, and D.A. Relman. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* 449:811-818.

Devaraj, N., M. Sheykhnazari, W.S. Warren, and V.P. Bhavanandan. 1994. Differential binding of Pseudomonas aeruginosa to normal and cystic fibrosis tracheobronchial mucins. . *Glycobiology.* 4:307–316.

Didierlaurent, A., J.-C. Sirard, J.-P. Kraehenbuhl, and M.R. Neutra. 2002. How the gut senses its content. *Cell Microbiol.* 4:61-72.

Drakes, M., T. Blanchard, and S. Czinn. 2004. Bacterial probiotic modulation of dendritic cells. *Infect Immun.* 72:3299-3309.

Dubuquoy, L., E.Å. Jansson, S. Deeb, S. Rakotobe, M. Karoui, J.-F. Colombel, J. Auwerx, S. Pettersson, and P. Desreumaux. 2003. Impaired expression of peroxisome proliferator-activated receptor γ in ulcerative colitis. *Gastroenterology* 124:1265-1276.

Duerkop, B.A., S. Vaishnava, and L.V. Hooper. 2009. Immune responses to the microbiota at the intestinal mucosal surface. *Immunity* 31:368-376.

Duerr, R.H. 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 314:1461-1463.

Eckburg, P.B., E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S.R. Gill, K.E. Nelson, and D.A. Relman. 2005. Diversity of the human intestinal microbial flora. *Science* 308:1635-1638.

Eglinton, B.A., D.M. Roberton, and A.G. Cummins. 1994. Phenotype of T cells, their soluble receptor levels, and cytokine profile of human breast milk. *Immunol Cell Biol* 72:306-313.

Engfer, M.B., B. Stahl, B. Finke, G. Sawatzki, and H. Daniel. 2000. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *Am J Clin Nutr* 71:1589-1596.

Fagarasan, S., and T. Honjo. 2003. Intestinal IgA synthesis: regulation of front-line body defences. *Nat Rev Immunol* 3:63-72.

Falk, P.G., L.V. Hooper, T. Midtvedt, and J. Gordon. 1998. Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol Mol Biol Rev..* 62:1157-1170.

Field, C.J. 2005. The immunological components of human milk and their effect on immune development in infants. *J Nutr.* 135:1-4.

Flemstrom, G., and M. Sjoblom. 2005. Epithelial cells and their neighbors. II. New perspectives on efferent signaling between brain, neuroendocrine cells, and gut epithelial cells. *Am J Physiol Gastrointest Liver Physiol.* 289:G377-380.

Franchimont, D. 2004. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. *Gut* 53:987-992.

Frey, A., K.T. Giannasca, R. Weltzin, P.J. Giannasca, H. Reggio, W.I. Lencer, and M.R. Neutra. 1996. Role of the glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: Implications for microbial attachment and oral vaccine targeting. *J Exp Med.* 184:1045-1059.

Fritz, J.H., R.L. Ferrero, D.J. Philpott, and S.E. Girardin. 2006. Nod-like proteins in immunity, inflammation and disease. *Nat Immunol* 7:1250-1257.

Gambaryan, A.S., A.B. Tuzikov, V.E. Piskarev, S.S. Yamnikova, D.K. Lvov, J.S. Robertson, N.V. Bovin, and M.N. Matrosovich. 1997. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl(N-acetyllactosamine). *Virology* 232:345–350.

Ganz, T. 2003. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 3:710-720.

Garofalo, R., S. Chheda, F. Mei, K.H. Palkowetz, H.E. Rudloff, F.C. Schmalstieg, D.K. Rassin, and A.S. Goldman. 1995. Interleukin-10 in human milk. *Pediatr Res.* 37:444-449.

Gewirtz, A.T., T.A. Navas, S. Lyons, P.J. Godowski, and J.L. Madara. 2001. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* 167:1882-1885.

Giannella, R.A., S.A. Broitman, and N. Zamcheck. 1972. Gastric acid barrier to ingested microorganisms in man: studies in vivo and in vitro. *Gut* 13:251-256.

Gibson, G.R., H.M. Probert, J.V. Loo, R.A. Rastall, and M.B. Roberfroid. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr Res Rev* 17:259-275.

Gibson, G.R., and M.B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr.* 125:1401-1412.

Gnoth, M.J., C. Kunz, E. Kinne-Saffran, and S. Rudloff. 2000. Human milk oligosaccharides are minimally digested in vitro. *J Nutr.* 130:3014-3020.

Gnoth, M.J., S. Rudloff, C. Kunz, and R.K.H. Kinne. 2001. Investigations of the in vitro transport of human milk oligosaccharides by a Caco-2 monolayer using a novel high performance liquid chromatography-mass spectrometry technique. *J Biol Chem.* 276:34363-34370.

Goldman, A.S. 1993. The immune system of human milk: antimicrobial, antiinflammatory and immunomodulating properties. *Pediatr Infect Dis J* 12:664-671.

Goldman, A.S. 2000. Modulation of the gastrointestinal tract of infants by human milk. Interfaces and interactions. An evolutionary perspective. *J Nutr.* 130:426-.

Gopal, P.K., and H.S. Gill. 2000. Oligosaccharides and glycoconjugates in bovine milk and colostrum. *Br J Nutr.* 84:69-74.

Halsey, J.F., C.S. Mitchell, and S.J. McKenzie. 1983. The origins of secretory IgA in milk: a shift during lactation from a serum origin to local synthesis in the mammary gland. *Ann N Y Acad Sci.* 409:452-460.

Hampe, J. 2007. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nature Genet.* 39:207-211.

Hanson, L. 1961. Comparative immunological studies of the immune globulins of human milk and blood serum. *Int Arch Allergy Appl Immunol.* 18:241-267.

Hanson, L. 1998. Breastfeeding provides passive and likely long-lasting active immunity. *Ann Allergy Asthma Immunol.* 81:523-533.

Hanson, L., F. Jalil, R. Ashraf, S. Bernin, B. Carlsson, J. Cruz, T. González, M. Hahn-Zoric, L. Mellander, and Y. Minoli. 1991. Characteristics of human milk antibodies and their effect in relation to the epidemiology of breastfeeding and infections in a developing country. *Adv Exp Med Biol* 310:1-15.

Hanson, L.Å., and M. Korotkova. 2002. The role of breastfeeding in prevention of neonatal infection. *Semin Neonatol.* 7:275-281.

Harmsen, H.J.M., A.C.M. Wildeboer-Veloo, G.C. Raangs, A.A. Wagendorp, N. Klijn, J.G. Bindels, and G.W. Welling. 2000. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr.* 30:61-67.

Harwig, S.S., L. Tan, X.D. Qu, Y. Cho, P.B. Eisenhauer, and R.I. Lehrer. 1995. Bactericidal properties of murine intestinal phospholipase A2. *J Clin Invest.* 95:603-610.

Hawkes, J.S., D.-L. Bryan, and R.A. Gibson. 2002. Cytokine production by human milk cells and peripheral blood mononuclear cells from the same mothers. *J Clin Immunol.* 22:338-344.

He, B., W. Xu, P.A. Santini, A.D. Polydorides, A. Chiu, J. Estrella, M. Shan, A. Chadburn, V. Villanacci, A. Plebani, D.M. Knowles, M. Rescigno, and A. Cerutti. 2007. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity* 26:812-826.

Hill, M.J. 1995. Bacterial fermentation of complex carbohydrate in the human colon. *Eur J Cancer Prev* 4:353-358.

Holzapfel, W.H., P. Haberer, R. Geisen, J. Bjorkroth, and U. Schillinger. 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am J Clin Nutr* 73:365S-373.

Hooper, L.V., T. Midtvedt, and J.I. Gordon. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr.* 22:283.

Hornef, M.W., T. Frisan, A. Vandewalle, S. Normark, and A. Richter-Dahlfors. 2002. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J Exp Med.* 195:559-570.

Hornef, M.W., B.H. Normark, A. Vandewalle, and S. Normark. 2003. Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells. *J Exp Med.* 198:1225-1235.

Horwitz, B.H. 2007. The straw that stirs the drink: Insight into the pathogenesis of inflammatory bowel disease revealed through the study of microflora-induced inflammation in genetically modified mice. *Inflamm Bowel Dis.* 13:490-500.

Hugenholtz, P., B.M. Goebel, and N.R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol.* 180:4765-4774.

Hugot, J.-P., M. Chamaillard, H. Zouali, S. Lesage, J.-P. Cezard, J. Belaiche, S. Almer, C. Tysk, C.A. O'Morain, M. Gassull, V. Binder, Y. Finkel, A. Cortot, R. Modigliani, P. Laurent-Puig, C. Gower-Rousseau, J. Macry, J.-F. Colombel, M. Sahbatou, and G. Thomas. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411:599-603.

Ichiba, H., S. Kusuda, Y. Itagane, K. Fujita, and G. Issiki. 1992. Measurement of growth promoting activity in human milk using a fetal small intestinal cell line. *Biol Neonate* 61:47-53.

Idota, T., and H. Kawakami. 1995. Inhibitory effects of milk gangliosides on the adhesion of Escherichia coli to human intestinal carcinoma cells. *Biosci Biotechnol Biochem.* 59:69–72.

Idota, T., H. Kawakami, Y. Murakami, and M. Sugawara. 1995. Inhibition of cholera toxin by human milk fractions and sialyllactose. *Biosci Biotechnol Biochem.* 59:417–419.

Inohara, N., M. Chamaillard, C. McDonald, and G. Nunez. 2005. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu Rev Biochem.* 74:355-383.

Isaacs, C.E. 2001. The antimicrobial function of milk lipids. *Adv Nutr Res.* 10:271.285.

Ivanov, I.I., R.d.L. Frutos, N. Manel, K. Yoshinaga, D.B. Rifkin, R.B. Sartor, B.B. Finlay, and D.R. Littman. 2008. Specific Microbiota Direct the Differentiation of IL-17-Producing T-Helper Cells in the Mucosa of the Small Intestine. *Cell Host Microbe.* 4:337-349.

Janeway, C.A. 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today* 13:11-16.

Kelly, D. 2004. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. *Nat Immunol.* 5:104-112.

Korzenik, J.R., and D.K. Podolsky. 2006. Evolving knowledge and therapy of inflammatory bowel disease. *Nat Rev Drug Discov* 5:197-209.

Kuntz, S., C. Kunz, and S. Rudloff. 2009. Oligosaccharides from human milk induce growth arrest via G2/M by influencing growth-related cell cycle genes in intestinal epithelial cells. *Br J Nutr.* 101:1306-1315.

Kuntz Sabine, S. Rudloff, and C. Kunz. 2008. Oligosaccharides from human milk influence growth-related characteristics of intestinally transformed and non-transformed intestinal cells. *Br J Nutr.* 99:462-471.

Kunz, C., S. Rudloff, W. Baier, N. Klein, and S. Strobel. 2000. Oligosaccharides in human milk: Structural, Functional, and Metabolic Aspects. *Annu Rev Nutr.* 20:699.

Labbok, M.H., D. Clark, and A.S. Goldman. 2004. Breastfeeding: maintaining an irreplaceable immunological resource. *Nat Rev Immunol* 4:565-572.

Labeta, M.O., K. Vidal, J.E.R. Nores, M. Arias, N. Vita, B.P. Morgan, J.C. Guillemot, D. Loyaux, P. Ferrara, D. Schmid, M. Affolter, L.K. Borysiewicz, A. Donnet-Hughes, and E.J. Schiffrin. 2000. Innate Recognition of Bacteria in Human Milk Is Mediated by a Milk-Derived Highly Expressed Pattern Recognition Receptor, Soluble Cd14. *J Exp Med.* 191:1807-1812.

Lasky, L.A. 1995. Selectin-carbohydrate interactions and the initiation of the inflammatory response. *Annu Rev Biochem.* 64:113-140.

LeBouder, E., J.E. Rey-Nores, A.-C. Raby, M. Affolter, K. Vidal, C.A. Thornton, and M.O. Labeta. 2006. Modulation of neonatal microbial recognition: TLR-mediated innate immune responses are specifically and differentially modulated by human milk. *J Immunol* 176:3742-3752.

LeBouder, E., J.E. Rey-Nores, N.K. Rushmere, M. Grigorov, S.D. Lawn, M. Affolter, G.E. Griffin, P. Ferrara, E.J. Schiffrin, B.P. Morgan, and M.O. Labeta. 2003. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. *J Immunol* 171:6680-6689.

Lee, J., D. Rachmilewitz, and E. Raz. 2006. Homeostatic Effects of TLR9 Signaling in Experimental Colitis. *Ann N Y Acad Sci.* 1072:351-355.

Ley, R.E., C.A. Lozupone, M. Hamady, R. Knight, and J.I. Gordon. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Micro* 6:776-788.

Ley, R.E., D.A. Peterson, and J.I. Gordon. 2006a. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124:837-848.

Ley, R.E., P.J. Turnbaugh, S. Klein, and J.I. Gordon. 2006b. Microbial ecology: Human gut microbes associated with obesity. *Nature* 444:1022-1023.

Lilly, D.M., and R.H. Stillwell. 1965. Probiotics: growth-promoting factors produced by microorganisms. *Science* 147:747-746.

Lönnerdal, B. 2010. Bioactive proteins in human milk: mechanisms of action. *J Pediatr.* 156:S26-S30.

Lönnerdal, B., and S. Lyer. 1995. Lactoferrin: molecular structure and biological function. *Annu Rev Nutr.* 15:93-110.

Looijer-Van Langen, M.A.C., and L.A. Dieleman. 2009. Prebiotics in chronic intestinal inflammation. *Inflamm Bowel Dis.* 15:454-462.

Louis, P., K.P. Scott, S.H. Duncan, and H.J. Flint. 2007. Understanding the effects of diet on bacterial metabolism in the large intestine. *J Appl Microbiol.* 102:1197-1208.

Macpherson, A.J., and N.L. Harris. 2004. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* 4:478-485.

Macpherson, A.J., L. Hunziker, K. McCoy, and A. Lamarre. 2001. IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms. *Microbes Infect.* 3:1021-1035.

Macpherson, A.J., and K. Smith. 2006. Mesenteric lymph nodes at the center of immune anatomy. *J Exp Med.* 203:497-500.

Macpherson, A.J., and T. Uhr. 2004. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303:1662-1665.

Matrosovich, M.N., A.S. Gambaryan, A.B. Tuzikov, N.E. Byramova, L.V. Mochalova, A.A. Golbraikh, M.D. Shenderovich, J. Finne, and B.N. V. 1993. Probing of the receptor-binding sites of the H1 and H3 influenza A and influenza B virus hemagglutinins by synthetic and natural sialosides. *Virology* 196111–121.

Maury, J., C. Nicoletti, L. Guzzo-Chambraud, and S. Maroux. 1995. The filamentous brush border glycocalyx, a mucin-like marker of enterocyte hyper-polarization. *Eur J Biochem.* 228:323-331.

Mazmanian, S.K., C.H. Liu, A.O. Tzianabos, and D.L. Kasper. 2005. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 122:107-118.

McCullough, J.S., B. Ratcliffe, N. Mandir, K.E. Carr, and R.A. Goodlad. 1998. Dietary fibre and intestinal microflora: effects on intestinal morphometry and crypt branching. *Gut* 42:799-806.

Medzhitov, R., and C.A. Janeway. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91:295-298.

Medzhitov, R., and J.C. Janeway. 2000. The Toll receptor family and microbial recognition. *Trends Microbiol.* 8:452-456.

Mehra, R., and P. Kelly. 2006. Milk oligosaccharides: Structural and technological aspects. *International Dairy Journal* 16:1334-1340.

Melmed, G., L.S. Thomas, N. Lee, S.Y. Tesfay, K. Lukasek, K.S. Michelsen, Y. Zhou, B. Hu, M. Arditi, and M.T. Abreu. 2003. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J Immunol* 170:1406-1415.

Meylan, E., and J. Tschopp. 2006. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol Cell.* 22:561-569.

Miller, J., S. Bull, J. Miller, and P. McVeagh. 1994. The oligosaccharide composition of human milk: temporal and individual variations in monosaccharide components. *J Pediatr Gastroenterol Nutr.* 19:371-376.

Moran, G.W., F.C. Leslie, S.E. Levison, and J.T. McLaughlin. 2008. Review: Enteroendocrine cells: Neglected players in gastrointestinal disorders? *Therapeutic Advances in Gastroenterology* 1:51-60.

Morrow, A.L., G.M. Ruiz-Palacios, M. Altaye, X. Jiang, M. Lourdes Guerrero, J.K. Meinzen-Derr, T. Farkas, P. Chaturvedi, L.K. Pickering, and D.S. Newburg. 2004. Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. *J Pediatr.* 145:297-303.

Mostov, K.E. 1994. Transepithelial transport of immunoglobulins. *Annu Rev Immunol.* 12:63-84.

Mowat, A., L. Parker, H. Beacock-Sharp, O.R. Millington, and F. Chirdo. 2004. Oral tolerance: overview and historical perspectives. *Ann N Y Acad Sci.* 1029:1-8.

Mowat, A.M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 3:331-341.

Mysore, J., P. Simon, D. Zopf, and A. Dubois. 1998. Treatment of Helicobacter Pylori infection in rhesus monkeys using a novel antiadhesion compound. *Gastroenterology* 114:A238-A238.

Neish, A.S. 2000. Prokaryotic Regulation of Epithelial Responses by Inhibition of IkB-a Ubiquitination. *Science* 289:1560-1563.

Neutra, M.R., N.J. Mantis, and J.-P. Kraehenbuhl. 2001. Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nat Immunol* 2:1004-1009.

Newburg, D.S. 1999. Human milk glycoconjugates that inhibit pathogens. . *Curr Med Chem.* 6:117–127.

Newburg, D.S. 2000. Are all human milks created equal? Variation in human milk oligosaccharides. *J Pediatr Gastroenterol Nutr.* 30:131-133.

Newburg, D.S. 2009. Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. *J Anim Sci.* 87:26-34.

Newburg, D.S., S. Ashkenazi, and T.G. Cleary. 1992. Human milk contains the Shiga toxin and Shiga-like toxin receptor glycolipid, Gb3. *J. Infect. Dis* 166:832–836.

Newburg, D.S., R.J. Linhardt, S.A. Ampofo, and Y.R. H. 1995. Human milk glycosaminoglycans inhibit HIV glycoprotein gp120 binding to its host cell CD4 receptor. *J Nutr.* 125:419–424.

Newburg, D.S., G.M. Ruiz-Palacios, and A.L. Morrow. 2005. Human milk glycans protect infants against enteric pathogens. *Annu Rev Nutr.* 25:37.

Newton, E.R. 2004. Breastmilk: the gold standard. *Clin Obstet Gynecol.* 47:632-642.

Obermeier, S., S. Rudloff, Pohlentz G, M. Lentze, and C. Kunz. 1999. Secretion of 13C-labelled oligosaccharides into human milk and infant's urine after an oral [13C]galactose load. *Isotopes Environ Health Stud.* 35:119-125.

Ogura, Y. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411:603-606.

Onai, N., M. Kitabatake, Y.-y. Zhang, H. Ishikawa, S. Ishikawa, and K. Matsushima. 2002. Pivotal role of CCL25 (TECK)-CCR9 in the formation of gut cryptopatches and consequent appearance of intestinal intraepithelial T lymphocytes. *Int Immunol.* 14:687-694.

Otnaess, A.B., A. Laegreid, and K. Ertresvag. 1983. Inhibition of enterotoxin from Escherichia coli and Vibrio cholerae by gangliosides from human milk. *Infect Immun.* 40:563–569.

Ouellette, A.J. 1999. IV. Paneth cell antimicrobial peptides and the biology of the mucosal barrier. *Am J Physiol.* 277:G257-261.

Perillo, N., M. Marcus, and L. Baum. 1998. Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. *J Mol Med.* 76:402-421.

Pitman, R.S., and R.S. Blumberg. 2000. First line of defense: the role of the intestinal epithelium as an active component of the mucosal immune system. *J Gastroenterol.* 35:805-814.

Podolsky, D.K. 1999. V. Innate mechanisms of mucosal defense and repair: the best offense is a good defense. *Am J Physiol.* 277:G495-499.

Podolsky, D.K. 2002. Inflammatory bowel disease. *N Engl J Med.* 347:417-429.

Potten, C., C. Booth, and D. Pritchard. 1997. The intestinal epithelial stem cell: the mucosal governor. *Int J Exp Pathol.* 78:219 243.

Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118:229-241.

Raybould, H.E. 2003. The Future of GI and Liver Research: Editorial Perspectives: IV. Visceral afferents: an update. *Am J Physiol Gastrointest Liver Physiol.* 284:G880-882.

Reiff, C., and D. Kelly. 2010. Inflammatory bowel disease, gut bacteria and probiotic therapy. *Int J Med Microbiol.* 300:25-33.

Rescigno, M., G. Rotta, B. Valzasina, and P. Ricciardi-Castagnoli. 2001. Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology* 204:572-581.

Resta-Lenert, S., and K.E. Barrett. 2006. Probiotics and commensals reverse TNF-a- and IFN-g-induced dysfunction in human intestinal epithelial cells. *Gastroenterology* 130:731-746.

Riley, M.A., and J.E. Wertz. 2003. Bacteriocins: evolution, ecology, and application. *Annu Rev Microbiol.* 56:117-137.

Rudloff, S., G. Pohlentz, L. Diekmann, H. Egge, and C. Kunz. 1996. Urinary excretion of lactose and oligosaccharides in preterm infants fed human milk or infant formula. *Acta Paediatr.* 85:598-603.

Rudloff, S., C. Stefan, G. Pohlentz, and C. Kunz. 2002. Detection of ligands for selectins in the oligosaccharide fraction of human milk. *Eur J Nutr.* 41:85-92.

Ruiz-Palacios, G.M., L.E. Cervantes, P. Ramos, B. Chavez-Munguia, and D.S. Newburg. 2003. Campylobacter jejuni binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem.* 278:14112-14120.

Saito, S., M. Yoshida, M. Ichijo, S. Ishizaka, and T. Tsujii. 1993. Transforming growth factor-beta (TGF-beta) in human milk. *Clin Exp Immunol.* 94:220-224.

Sajjan, S.U., and J.F. Forstner. 1990. Role of the putative "link" glycopeptide of intestinal mucin in binding of piliated Escherichia coli serotype O157:H7 strain CL-49. *Infect Immun.* 58:868-873.

Sallusto, F., and A. Lanzavecchia. 2009. Heterogeneity of $CD4^+$ memory T cells: Functional modules for tailored immunity. *Eur J Immunol.* 39:2076-2082.

Sartor, R.B. 2004. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* 126:1620-1633.

Sartor, R.B. 2008. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 134:577-594.

Savage, D.C. 1977. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol.* 31:107-133.

Savage, D.C. 1986. Gastrointestinal microflora in mammalian nutrition. *Annu Rev Microbiol.* 6:155-178.

Savage, D.C., J.E. Siegel, J.E. Snellen, and D.D. Whitt. 1981. Transit time of epithelial cells in the small intestines of germfree mice and ex-germfree mice associated with indigenous microorganisms. *Appl Environ Microbiol.* 42:996-1001.

Schroten, H., F. Hanisch, R. Plogmann, J. Hacker, G. Uhlenbruck, R. Nobis-Bosch, and V. Wahn. 1992. Inhibition of adhesion of S-fimbriated Escherichia coli to buccal epithelial cells by human milk fat globule membrane components: a novel aspect of the protective function of mucins in the nonimmunoglobulin fraction. *Infect Immun.* 60:2893-2899.

Sela, D.A., and D.A. Mills. 2010. Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides. *Trends Microbiol.* In Press, Corrected Proof:

Selsted, M.E., and A.J. Ouellette. 2005. Mammalian defensins in the antimicrobial immune response. *Nat Immunol* 6:551-557.

Sharon, N. 1996. Carbohydrate-lectin interactions in infectious disease. *Adv Exp Med Biol.* 408:1-8.

Shen, L., and J.R. Turner. 2006. Role of Epithelial Cells in Initiation and Propagation of Intestinal Inflammation. Eliminating the static: tight junction dynamics exposed. *Am J Physiol Gastrointest Liver Physiol* 290:G577-582.

Sherman, P.M., M. Cabana, G.R. Gibson, B.V. Koletzko, J. Neu, G. Veereman-Wauters, E.E. Ziegler, and W.A. Walker. 2009. Potential roles and clinical utility of prebiotics in newborns, infants, and children: proceedings from a global prebiotic summit meeting, New York City, June 27-28, 2008. *J Pediatr.* 155:S61-S70.

Silverberg, M.S. 2007. Refined genomic localization and ethnic differences observed for the IBD5 association with Crohn's disease. *Eur J Hum Genet.* 15:328-335.

Sokol, H., C. Lay, P. Seksik, and G.W. Tannock. 2008. Analysis of bacterial bowel communities of IBD patients: What has it revealed? *Inflamm Bowel Dis.* 14:858-867.

Specian, R.D., and M.G. Oliver. 1991a. Functional biology of intestinal goblet cells. *Am J Physiol Cell Physiol* 260:C183-193.

Specian, R.D., and M.G. Oliver. 1991b. Functional biology of intestinal goblet cells. . *Am J Physiol Cell Physiol* 260:C183-193.

Stappenbeck, T.S., L.V. Hooper, and J.I. Gordon. 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proc Natl Acad Sci U S A.* 99:15451-15455.

Stehle, T., Y. Yan, T.L. Benjamin, and H.S. C. 1994. Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature* 369:160–163.

Stins, M.F., N.V. Prasadarao, L. Ibric, C.A. Wass, P. Luckett, and, and K.K. S. 1994. Binding characteristics of S-fimbriated Escherichia coli to isolated brain microvascular endothelial cells. *Am J Pathol* 145:1228–1236.

Strobel, S., and A.M. Mowat. 2006. Oral tolerance and allergic responses to food proteins. *Curr Opin Allergy Clin Immunol.* 6:207-213

Tao, N., E.J. DePeters, S. Freeman, J.B. German, R. Grimm, and C.B. Lebrilla. 2008. Bovine Milk Glycome. *J Dairy Sci.* 91:3768-3778.

Thurl, S., J. Henker, M. Siegel, K. Tovar, and G. Sawatzki. 1997. Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. *Glycoconj J.* 14:795-799.

Turner, J.R. 2009. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 9:799-809.

Urashima, T., S. Murata, and T. Nakamura. 1997. Structural determination of monosialyl trisaccharides obtained from caprine colostrum. *Comp Biochem Physiol B Biochem Mol Biol* 116:431-435.

Urashima, T., T. Saito, T. Nakamura, and M. Messer. 2001. Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconj J.* 18:357-371.

Vaishnava, S., C.L. Behrendt, A.S. Ismail, L. Eckmann, and L.V. Hooper. 2008. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci U S A* 105:20858-20863.

van Wijk, F., and H. Cheroutre. 2009. Intestinal T cells: Facing the mucosal immune dilemma with synergy and diversity. *Semin Immunol.* 21:130-138.

Varki, A. 1994. Selectin ligands. *Proc Natl Acad Sci USA* 91:7390-7397.

Varki, A. 1997. Selectin ligands: will the real ones please stand up? *J Clin Invest* 100:S31-35.

Varki, A., and T. Angata. 2006. Siglecs--the major subfamily of I-type lectins. *Glycobiology* 16:1R-27.

Veckman, V., M. Miettinen, J. Pirhonen, J. Siren, S. Matikainen, and I. Julkunen. 2004. Streptococcus pyogenes and Lactobacillus rhamnosus differentially induce maturation and production of Th1-type cytokines and chemokines in human monocyte-derived dendritic cells. *J Leukoc Biol* 75:764-771.

Vesterlund, S., J. Paltta, A. Lauková, M. Karp, and A.C. Ouwehand. 2004. Rapid screening method for the detection of antimicrobial substances. *J Microbiol Methods* 57:23-31.

Vignali, D.A.A., L.W. Collison, and C.J. Workman. 2008. How regulatory T cells work. *Nat Rev Immunol* 8:523-532.

Virkola, R., J. Parkkinen, J. Hacker, and T.K. Korhonen. 1993. Sialyloligosaccharide chains of laminin as an extracellular matrix target for S fimbriae of Escherichia coli. *Infect Immun.* 61:4480–4484.

Viverge, D., L. Grimmonprez, G. Cassanas, L. Bardet, and M. Solere. 1990. Discriminant carbohydrate components of human milk according to donor secretor types. *J Pediatr Gastroenterol Nutr.* 11:365-370.

Viveros-Rogel, M., L. Soto-Ramirez, P. Chaturvedi, D.S. Newburg, and G.M. Ruiz-Palacios. 2004. Inhibition of HIV-1 infection in vitro by human milk sulfated glycolipids and glycosaminoglycans. *Adv Exp Med Biol.* 554:481–487.

Wald, D., J. Qin, Z. Zhao, Y. Qian, M. Naramura, L. Tian, J. Towne, J.E. Sims, G.R. Stark, and X. Li. 2003. SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. *Nat Immunol* 4:920-927.

Wang, X., and R. Andersson. 1994. Intestinal Brush Border Membrane Function. *Scand J Gastroenterol.* 29:289-299.

Weaver, C.T., R.D. Hatton, P.R. Mangan, and L.E. Harrington. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol.* 25:851-852.

Wehkamp, J., J. Harder, K. Wehkamp, B.W.-v. Meissner, M. Schlee, C. Enders, U. Sonnenborn, S. Nuding, S. Bengmark, K. Fellermann, J.M. Schroder, and E.F. Stange. 2004. NF-kB- and AP-1-Mediated Induction of Human B Defensin-2 in Intestinal Epithelial Cells by Escherichia coli Nissle 1917: a Novel Effect of a Probiotic Bacterium. *Infect Immun.* 72:5750-5758.

Wehkamp, J., N.H. Salzman, E. Porter, S. Nuding, M. Weichenthal, R.E. Petras, B. Shen, E. Schaeffeler, M. Schwab, R. Linzmeier, R.W. Feathers, H. Chu, H. Lima, K. Fellermann, T. Ganz, E.F. Stange, and C.L. Bevins. 2005. Reduced Paneth cell bdefensins in ileal Crohn's disease. *Proc Natl Acad Sci USA* 102:18129-18134.

Weis, W., M. Taylor, and K. Drickamer. 1998. The C-type lectin superfamily in the immune system. *Immunol Rev.* 163:19-34.

Wen, L., R.E. Ley, P.Y. Volchkov, P.B. Stranges, L. Avanesyan, A.C. Stonebraker, C. Hu, F.S. Wong, G.L. Szot, J.A. Bluestone, J.I. Gordon, and A.V. Chervonsky. 2008. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* 455:1109-1113.

Whitman, W.B., D.C. Coleman, and W.J. Wiebe. 1998. Prokaryotes: The unseen majority. *Proc Natl Acad Sci USA* 95:6578-6583.

Worbs, T., U. Bode, S. Yan, M.W. Hoffmann, G. Hintzen, G. Bernhardt, R. Förster, and O. Pabst. 2006. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J Exp Med* 203:519–527.

Wostmann, B.S. 1981. The germfree animal in nutritional studies. *Annu Rev Nutr.* 1:257.

Zhang, G., and S. Ghosh. 2002. Negative regulation of toll-like receptor-mediated signaling by Tollip. *J Biol Chem.* 277:7059-7065.

Zhou, L., M.M.W. Chong, and D.R. Littman. 2009. Plasticity of CD4+ T cell lineage differentiation. *Immunity* 30:646-655.

Zyrek, A.A., C. Cichon, S. Helms, C. Enders, U. Sonnenborn, and M.A. Schmidt. 2007. Molecular mechanisms underlying the probiotic effects of Escherichia coli Nissle 1917 involve ZO-2 and PKCzeta redistribution resulting in tight junction and epithelial barrier repair. *Cell Microbiol.* 9:804-816.

Results

Milk sialyllactose influences colitis in mice through selective intestinal bacterial colonization

Andrea Fuhrer[1], Norbert Sprenger[2], Lubor Borsig[1], Christophe Chassard[3], Thierry Hennet[1]

[1]Institute of Physiology and Center for Integrative Physiology, University of Zurich, Switzerland, [2]Nestlé Research Center, Vers-chez-les-Blanc, Lausanne, Switzerland, [3]Laboratory of Food Biotechnology, Institute of Food, Nutrition and Health, ETH Zurich, Switzerland.

Correspondence should be sent to: Thierry Hennet
Institute of Physiology, University of Zürich,
Winterthurerstrasse 190
CH-8057 Zürich, Switzerland
Tel: +41 44 635-5080
Fax: +41 44 635-6814
thennet@access.uzh.ch

Running title: Role of milk sialyllactose in DSS-induced colitis

Character count (except M&M and References): 24,267 (not including spaces)

Abstract

Milk oligosaccharides contribute to the development of the intestinal environment by acting as decoy receptors for pathogens and as prebiotics, which promote the colonization of commensal bacteria. The role of the sialylated milk oligosaccharides sialyl(α2,3)lactose and sialyl(α2,6)lactose on mucosal immunity was investigated in α2,3- and α2,6-sialyltransferase-null mice deficient for one type of milk sialyllactose. The exposure of newborn mice to milk containing or deficient for sialyllactose had no impact on the development of mucosal leukocyte populations. However, when adult mice were challenged by dextran sulfate sodium (DSS) in drinking water, mice previously exposed to sialyl(α2,3)lactose-deficient milk were more resistant to colitis compared to mice exposed to normal milk and sialyl(a2,6)lactose-deficient milk. The analysis of intestinal microbiota showed different colonization patterns depending on the presence or absence of sialyl(α2,3)lactose in the milk. The relationship between intestinal microbiota and the severity of DSS-induced colitis was established by showing that germfree mice reconstituted with intestinal microbiota isolated from mice fed on sialyl(α2,3)lactose-deficient milk were more resistant to DSS-induced colitis than germfree mice reconstituted with standard intestinal microtiota. The study demonstrated that the exposure to sialyllactose during infancy affects the bacterial colonization of the intestine, which directly influences the susceptibility to DSS-induced colitis in adult mice.

Introduction

Oligosaccharides represent a major fraction of milk constituents. Unique among mammals, human milk contains a tremendous diversity of oligosaccharide structures, which are shaped by extension of lactose through glycosyltransferase enzymes in the mammary gland (Egge, 1993). The most abundant structures are the trisaccharides produced by addition of fucose or sialic acid to lactose. Whereas fucosylated oligosaccharides are missing from most mammalian milks, sialylated oligosaccharides are more widely distributed (Urashima et al., 2001). Since milk oligosaccharides are neither digested, nor absorbed in the small intestine (Brand Miller et al., 1995), they have been suggested to contribute to the development of the infant gastrointestinal tract and its colonization by commensal bacteria (Frank and Pace, 2008; Savage, 1977).

Milk oligosaccharides influence the development of the intestinal microbiota by acting as selective nutrients, which support the proliferation of specific bacterial groups (Gibson and Roberfroid, 1995). The prebiotic action of milk oligosaccharides has been demonstrated by comparing the intestinal microbiota of infants fed on oligosaccharide-rich breast milk and infants fed on formula (Harmsen et al., 2000). Furthermore, considering the structural similarity of milk oligosaccharides with cell surface glyans, milk oligosaccharides can function as soluble receptors, thereby preventing the attachment of pathogenic bacteria to intestinal epithelial cells (Newburg, 2009).

Commensal bacteria are mainly found in the large intestine, consisting predominantly of the phyla *Firmicutes* and *Bacteroidetes* (Eckburg et al., 2005). *Firmicutes* themselves are composed of two major clostridial groups, namely the clostridial cluster IV and clostridial cluster XIVa, which comprises *Lachnospiraceae*. The density and diversity of the intestinal microbiota are highly complex. Culture-independent methods have allowed to estimate the presence of 500-1000 different species and over 7000 strains in the human gastrointestinal tract (Dethlefsen et al., 2007; Ley et al., 2006). Commensal bacteria are indispensible for the proper development of the mucosal immune system. In addition to morphological development of immune compartments, bacterial colonization initiates antibody production (Macpherson and Harris, 2004) and the production of antimicrobial proteins such as defensins (Falk et al., 1998). Bacterial cell wall components have also been shown to regulate $CD4^+$ T helper cell activity in the *lamina propria* (Gaboriau-Routhiau et al., 2009). On the other hand, a defect of the innate immune system has been shown to affect the composition of the intestinal microbiota in mice and thereby contributes to metabolic imbalance (Vijay-Kumar et al., 2010). In humans, similar relationships have been

established between the intestinal microbiota and energy balance, thus leading to the definition of microbiomes typical for disorders such as obesity (Turnbaugh et al., 2006).

Prebiotic oligosaccharides have been claimed to reduce the susceptibility to allergies in infants (Hoffen et al., 2009; Moro et al., 2006) and shown to influence the immune response to vaccination in mice (Vos et al., 2007). However, the mechanisms underlying the regulatory action of oligosaccharides remain largely unknown. Considering the structural complexity of human milk oligosaccharides, the mouse with its reduced range of milk oligosaccharides (Prieto et al., 1995) enables addressing the functional impact of specific oligosaccharides on mucosal immunity *in vivo*. In the present study, we have studied the impact of sialylated oligosaccharides using sialyltransferase-knockout mice deficient for these milk oligosaccharides. The fostering of newborn mice by normal or sialyllactose-deficient mothers allowed demonstrating the importance of this type of milk oligosaccharides on the colonization of intestinal bacteria and on the susceptibility to an experimental colitis model.

Results

The sialyltransferase enzymes St6gal1 and St3gal4 are responsible for the production of sialyllactose in mouse milk.

The composition of mouse milk oligosaccharides is limited to sialyllactoses with only traces of fucosylated lactose (Kuhn, 1972; Prieto et al., 1995). This low structural complexity makes the mouse a suitable model to investigate the role of these specific milk oligosaccharides on mucosal immunity. Because multiple α2,3 and α2,6 sialyltransferase enzymes (Harduin-Lepers et al., 2001) could be responsible for the production of sialyllactose, we have first examined the expression of these genes in the lactating mammary gland. The mRNA levels of the α2,3 sialyltransferase genes *St3gal1* to *St3gal6* and of the two α2,6 sialyltransferase genes *St6gal1* and *St6gal2* were determined by real-time PCR . The expression of the *St6gal1* gene was induced up to 20-fold during lactation, suggesting this sialyltransferase may account for the biosynthesis of sialyl(α2,6)lactose (6SL) (Fig. 1) and thus confirming a previously published observation (Dalziel et al., 2001). Among α2,3 sialyltransferase genes, the expression of *St3gal1* and *St3gal4* were induced by 3- and 4-fold during lactation, respectively (Fig. 1). The three *St6gal1, ST3gal1* and *St3gal4* represented also the most abundant sialyltransferase transcripts in lactating mammary gland when mRNA levels were normalized to GAPDH (data not shown).

To confirm the involvement of the *St3gal1*, *St3gal4* and *St6gal1* sialyltransferases in the production of milk sialyllactose, the oligosaccharide composition of milk isolated from *St3gal1*, *St3gal4* and *St6gal1* sialyltransferase-deficient mice was determined by pulsed amperometry-HPAEC. Milk isolated from *St3gal1*-null mice showed unaffected or rather increased levels of both sialyl(α2,3)lactose (3SL) and 6SL when compared to the levels measured in wildtype (Wt) mice by day two of lactation (Fig. 2A). The analysis of oligosaccharides in milk from *St3gal4*-null mice showed a strong decrease of 3SL, indicating that this sialyltransferase accounts for the bulk of 3SL production. The importance of *St6gal1* in the production of 6SL was confirmed by the absence of this oligosaccharide in the milk isolated from *St6gal1*-null mice (Fig. 2A). The impact of *St3gal4* on 3SL production was investigated in more details by measuring sialyllactose levels across lactation. In Wt mice, 3SL levels peaked in the first week of lactation and slowly decreased until day 20, where only minor concentrations were detected. By contrast, 6SL levels showed only a modest increase by mid-lactation (Fig. 2B). The 3SL peak by the first week of lactation was absent in the milk of *St3gal4*-null mice and 3SL levels remained low across lactation, thus demonstrating the importance of *St3gal4* for the biosynthesis of 3SL (Fig. 2C). The identity of 3SL and 6SL and exact structure of sialic acid in mouse milk were also analyzed

after release by neuraminidase treatment. We did find that milk sialyllactose was exclusively composed of N-acetylneuraminic acid since no N-glycolylneuraminic acid could be detected (data not shown).

Feeding with milk deficiency in 3SL increases the resistance of mice to dextran sulfate sodium-induced colitis

The role of milk 3SL and 6SL onto the development of the mucosal immune system was addressed by feeding Wt and *St3gal4*-null newborn mice with normal or with 3SL-deficient milk as achieved by cross-fostering litters with Wt and *St3gal4*-null mothers. The same cross-fostering approach was applied to study the role of 6SL. Leukocyte populations and IgA secretion were determined in three-, six- and twelve-week old mice by flow cytometry and enzyme-linked immunosorbent assay, respectively. The T-cell specific markers TCRαβ/γδ, CD4, CD8 and CD8αα/αβ were measured in Wt, *St3gal4*-null, *St6gal1*-null and correspondingly cross-fostered mice. No differences were noticeable for leukocyte populations and IgA secretion, indicating that neither the feeding with sialyllactose-deficient milk, nor the disruption of the *St3gal4* and *St6gal1* genes had any impact on the maturation of intestinal leukocytes.

In a second approach, we have addressed whether feeding with 3SL- and 6SL-deficient milk affected the response of mice to an intestinal challenge. At seven weeks of age, mice were exposed to dextran sulfate sodium (DSS) in drinking water for five days. DSS impairs the integrity of intestinal barrier, thereby inducing an acute colitis (Okayasu I, 1990). Wt mice responded strongly to the treatment as shown by a loss of body weight of 17% by day seven. By contrast, *St3gal4*-null mice were more resistant to DSS as they lost only 5% of their body weight by day seven (Fig. 3A). Wt mice that were cross-fostered and fed with 3SL-deficient milk sustained the DSS treatment better than those fed with normal milk, as shown by a reduced loss of body weight of 8%. Correspondingly, *St3gal4*-null mice fed with normal milk were more susceptible to DSS-induced colitis than littermates fed with 3SL-deficient milk (Fig. 3A). DSS-induced colitis in *St6gal1*-null mice lead to a similar disease as found in Wt mice. Accordingly, cross-fostering experiments with normal and 6SL-deficient milk had no impact on the susceptibility to acute colitis (data not shown). We therefore focused on the investigation of 3SL in colitis development.

The severity of colitis was also registered by measuring colon length and epithelial permeability. At day seven, colon length was shortened by 20-25% in Wt mice, whereas it was shortened by 10% in *St3gal4*-null mice (Fig. 3B). Cross-fostered mice showed a degree of colon shortening

that matched the loss of body weight. Wt mice fed with 3SL-deficient milk showed only a 10% colon shortening and *St3gal4*-null mice fed with normal milk showed a more pronounced colon shortening (Fig. 3B). A similar picture was obtained by examining epithelial permeability in the intestine. DSS treatment increased epithelial leakiness in Wt mice but not in *St3gal4*-null mice. The finding was reversed when looking at cross-fostered Wt and *St3gal4*-null mice fed with 3SL-deficient and normal milk, respectively (Fig. 3C). The severity of colitis was also appreciated by measuring the inflammatory cytokines TNFα, IL-1β and IL-6. The levels of TNFα and IL-1β expression were not elevated by day seven but IL-6 expression was induced 59-fold in mice, which had been fed with normal milk, whereas IL-6 expression was less pronounced, around 25-fold, in mice exposed to 3SL-deficient milk (Fig. 3D-F).

The extent and types of cells infiltrating the inflamed colons were analyzed by flow cytometry in Wt, *St3gal4*-null mice and correspondingly cross-fostered mice. By day five, 27 to 40% of cells recovered from the *lamina propria* were $CD45^+$ (Fig. 4). By day 7, additional 5-10% CD45-positive cells were detected (Fig. 4A). Leukocyte infiltration was highest in mice fed with normal milk, but infiltration was only slightly decreased in mice that had been fed with 3SL-deficient milk (Fig. 4B). The $CD45^+$ cells were identified as B cells (68%, $CD19^+$) and granulocytes (20%, $Gr1^+$) with a minor population of T cells (7%, $CD3^+$). The contribution of *St3gal4*-null leukocytes to the resistance towards DSS-induced colitis was tested on irradiated Wt mice transplanted with *St3gal4*-null bone marrow and irradiated *St3gal4*-null mice transplanted with Wt bone marrow. After transplantation, mice were given seven weeks to recover from mucosal injury sustained during irradiation before being subjected to DSS treatment. Wt mice bearing immune cells from *St3gal4*-null mice rapidly developed colitis and reached the critical weight loss of 15% whereas *St3gal4*-null mice with Wt leukocytes showed only 3% weight loss (Fig. 4C). We thus concluded that the resistance to DSS-induced colitis was independent of the leukocyte genotype.

Feeding with 3SL-deficient milk modifies the bacterial colonization of the mouse intestine

We did show so far that the exposition to milk in the first three weeks of life had an impact on the susceptibility to DSS-induced colitis tested in adult mice. Considering the known effect of milk oligosaccharides as prebiotics (Bode, 2009), we addressed whether the presence or absence of 3SL affected the composition of the intestinal microbiota and thereby the outcome of DSS-induced colitis. To address the first point, we fingerprinted the intestinal microbiota of Wt, *St3gal4*-null and cross-fostered mice by temporal temperature gradient gel electrophoresis (Livak and Schmittgen) and by real-time PCR. TTGE fingerprints from each six mice of each group were analyzed at three, six (Fig. 5A) and twelve weeks of age. Despite internal differences

within groups, cluster analysis indicated a higher degree of similarity within than between these groups. A specific band, identified by sequencing as representing a *Ruminococcaceae* species, was only identified in the microbiota of mice exposed to 3SL, namely in Wt mice and in *St3gal4*-null mice fed with Wt milk (Fig. 5A). The sequence obtained from the TGGE band indicated that the *Ruminococcaceae* species was very close to Ruminococcaceae from the clostridial cluster IV, yet different from the species known to date. By comparison, the presence or absence of 6SL as tested with *St6gal1*-null mice did not affect the occurrence of the *Ruminococcaceae* in the intestinal microbiota (data not shown), indicating that these bacteria require 3SL for gut colonization.

The diversity of the intestinal microbiota was also determined during DSS-induced colitis. A lower amount of bands on the gels were detected in DSS-treated mice compared to healthy animals indicating a decrease of microbial diversity (Fig. 5B). The band distribution on the gel was also shifted in mice treated with DSS, showing a change in microbial composition. Interestingly the *Ruminococcaceae*-related band remained unchanged during colitis. The real-time PCR analysis of the five phylogenic groups *Enterobacteriaceae*, *Lachnospiraceae*, clostridial cluster IV, *Bacteroidetes* and *Lactobacillaceae* in Wt, *St3gal4*-null and cross-fostered mice confirmed that 3SL deficiency in the milk only affected the colonization of clostridial cluster IV bacteria (Fig. 6A). However, during DSS-induced colitis, the previous exposure to 3SL in milk influenced the relative composition of *Enterobacteriaceae* and the clostridial cluster IV to total bacteria. *Enterobacteriaceae* and the clostridial cluster IV were more abundant in Wt and cross-fostered *St3gal4*-null mice after DSS treatment (Fig. 6B).

Considering the differential detection of a *Ruminococcaceae* species by TTGE analysis and the fact that several *Ruminococcaceae* are part of the clostridial cluster IV (Van Dyke and McCarthy, 2002), we have measured the abundance of these bacteria using PCR primers that specifically target 16S rRNA from *Ruminococcus* genera. In agreement with the TTGE data, *Ruminococcaceae* were more abundant in mice that had been exposed to 3SL containing milk, i.e. in Wt and cross-fostered *St3gal4*-null mice (Fig. 6C). The treatment with DSS did not alter the levels of *Ruminococcaceae*, thereby confirming the TTGE findings (Fig. 5B).

Microbiota isolated from mice fed with 3SL-deficient milk increase the resistance of reconstituted germfree mice to DSS-induced colitis

To demonstrate that the effect of 3SL-deficiency towards DSS treatment was mediated by selective bacterial colonization of the intestine, we have reconstituted germfree mice with

intestinal microbiota isolated from the cecum of Wt and St3gal4-null mice. Colonization success was confirmed by TTGE analysis of fecal pellets two weeks after reconstitution. The microbiota from Wt mice included the *Ruminococcus*-related specific band, whereas the microbiota from *St3gal4*-null mice lacked this major band (Fig. 7A). The mice with reconstituted microbiota were then subjected to DSS treatment by twelve weeks of age. The mice with the Wt derived microbiota showed a progressive loss of body weight similar to Wt mice, reaching 82% of their initial body weight by day seven (Fig. 7B). By contrast, the mice with the *St3gal4*-null derived microbiota showed a moderate weight loss to 90% of initial body weight, thus demonstrating that the composition of the intestinal microbiota had a direct influence on the susceptibility to DSS-induced colitis.

Discussion

The present study demonstrated the impact of the milk sialyllactose 3SL on the colonization of the murine intestinal microbiota and thereby on the susceptibility to DSS-induced colitis. The application of the cross-fostering setup between Wt and *St3gal4*-null mice demonstrated for the first time that a single oligosaccharide structure influences microbial composition *in vivo*. The different abundance of specific bacterial groups like *Ruminococcaceae* in mice fed with normal or 3SL-deficient milk correlated with the susceptibility to DSS-induced colitis. The first question arising when considering the model investigated is whether the oligosaccharide 3SL exerts regulatory functions on the mucosal immune system, thereby influencing the immune response to DSS exposure. Our survey of leukocyte populations did not reveal any differences between Wt, *St3gal4*-null and cross-fostered mice, thus speaking against such an immunoregulatory effect of 3SL. Furthermore, the bone marrow transplantation between Wt and *St3gal4*-null mice showed that the susceptibility to DSS did not correlate with the genotype of leukocytes. Finally, the differential susceptibility of reconstituted germfree mice to DSS demonstrated that the effect of 3SL-deficient milk was mediated by microbiota and not by the mucosal immune system.

The exposure to 3SL during lactation could influence the colonization of intestinal bacteria by affecting their adhesion to the intestinal epithelium or by serving as nutrients for specific groups of bacteria. Milk 3SL could impair the attachment of bacteria binding to sialylated surfaces (Sakarya et al., 2003) or induce phase variation, thereby decrease type 1 fimbriae expression on some bacteria (Sohanpal et al., 2004). However, we could not address such an effect of 3SL on the colonization of *Ruminococcaceae*, since we did not succeed yet at isolating this bacterial group in culture. Alternatively, 3SL could be used as a carbon and nitrogen source, which would facilitate the proliferation of bacteria capable of metabolizing sialic acid, as shown for the intestinal colonization of *E. coli* and *V. cholera* (Almagro-Moreno and Boyd, 2009b; Chang et al., 2004). Noteworthy, the Nan-cluster of genes required for the catabolism of sialic acid is found in several pathogenic and commensal bacteria including *Ruminococcus gnavus* (Almagro-Moreno and Boyd, 2009a). Thus the selective colonization of *Ruminococcaceae* in the presence of 3SL might be mediated through their ability to use 3SL for energy gain.

The gram-positive *Ruminococcaceae* are obligate anaerobes that are commonly found in the colon of mammals including mice and humans (Collins et al., 1994). *Ruminococcaceae* are known to ferment polysaccharides like cellulose and starch (Herbeck and Bryant, 1974; Leitch et al., 2007; Wang et al., 1997). The correlation between the abundance of the *Ruminococcus*-related species and the susceptibility to DSS-induced colitis suggests that this bacterium may

have a pro-inflammatory action. The fact that *Ruminococcaceae* have been found enriched in patients with inflammatory bowel disease (Andoh et al., 2007; Martinez-Medina et al., 2006; Pirindiville et al., 2004) supports their potential role as pro-inflammatory bacteria. Moreover, the intestinal colonization of *Ruminococcaceae* may impair the settlement of other bacterial groups, which are known to attenuate the extent of the inflammatory response (Im et al., 2009; Kumar et al., 2008)

At the present stage, it is not possible to demonstrate a direct relationship between the intestinal abundance of *Ruminococcaceae* and the severity of DSS-induced colitis. The isolation and culture of *Ruminococcaceae* in vitro would allow the selective reconstitution of germfree mice with these bacteria and subsequently to address the susceptibility of the mice to DSS treatment. We are currently trying to enrich for *Ruminococcaceae* on 3SL-containing media in order to assess their exact role in the development of colitis.

Our study has demonstrated that the exposure to a single milk oligosaccharide structure can significantly influence intestinal bacterial colonization and thereby affect the susceptibility of the host to DSS-induced colitis. The fact that mice fed with 3SL-deficient milk were more resistant to DSS treatment is somehow paradoxical, considering that this oligosaccharide is evolutionary conserved in most mammals. Although 3SL has a pro-inflammatory effect in the DSS model, it can be assumed that 3SL may mediate protective actions, as for example by preventing the adhesion of pathogenic viruses and bacteria during infancy. Further, the 3SL dependent microbiota might confer an evolutionary advantage by promoting an inflammatory defense reaction upon an infection challenge. The fact that 3SL levels in milk are elevated in the first days postpartum and strongly decrease until weaning may indicate the need for a balanced availability of the oligosaccharide in the developing gastrointestinal tract. The study of additional immunological challenges and infectious models in sialyltransferase-null mice will further clarify the biological importance of sialylated milk oligosaccharides in the physiology of the gastrointestinal tract.

Matherials and methods

Mouse models - Sialyltransferase *St6gal1*-null (Hennet et al., 1998), *St3gal1*-null (Priatel et al., 2000) and *St3gal4*-null (Ellies et al., 2002) were provided by Dr. Jamey Marth (University of California Santa Barbara, USA). All mice were in the C57Bl/6 background. Sialyltransferase-null and Wt control mice were housed in light-cycled and climate-controlled rooms. All experiments were performed in compliance with the Swiss Animal Protection Ordinance and approved by the local veterinary authority (Kantonales Veterinäramt Zürich, Switzerland). Synchronized matings were setup for sialyltransferase and Wt control mice to allow the exchange of newborn mice for cross-fostering experiments. To this end both the mothers and the litter of the other genotype were transferred to new cages including parts of the mother's nests.

Sialyltransferase gene expression in mammary glands - Commercial RNA (Axxora Ltd., UK) isolated from mammary glands of virgin mice and mice after one and two weeks postpartum and three days post weaning was used to monitor sialyltransferase expression profiles by real-time PCR using a SybrGreen protocol. Briefly, RNA samples were treated with DNase (DNA-Free, Ambion, USA) according to manufacturer's instructions. Reverse transcription (RT) reactions were performed with 2 µg of total RNA and random hexamer primers using the Thermoscript RT-PCR System (Invitrogen, USA) according to manufacturer's instructions. Real-time PCR reactions were performed using the SYBR Green PCR Master Mix (Applied Biosystems, USA) of 24 µl and 1 µl of test cDNA per reaction. The primers used for amplification of mouse sialyltransferase genes are given in table 1. After an initial denaturation step of 10 min at 95°C, 40 cycles at 95°C for 15 s, 60°C for 1 min and 72°C for 1 min. Gene expression was normalized to GAPDH expression calculations were done according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Mouse milking - Lactating mothers were separated from their suckling newborns for four hours. Milk ejection was stimulated by intraperitoneal injection of 0.5 IU of oxytocin (Sigma, Switzerland). Mice were anesthetized by ketamine (0.65 ml/kg) and xylazine (0.5 ml/kg). Milk was collected by aspiration (Haberman, 1974; Nagasawa, 1979), then frozen and lyophilized. Dry matter was determined by weighing and samples were resuspended in water to yield a stock solution of 100 mg/ml.

Milk oligosaccharide analysis - For each sample an equivalent of 16.5 µg dry matter was separated on a CarboPac PA200 analytical column (Dionex, Sunnyvale, CA, USA) with an aminotrap guard (Dionex) using a high performance anion exchange chromatography system

ICS3000 (Dionex) equipped with a pulsed amperometry detector. The column compartment was set to 25°C and the flow speed to 0.38 ml/min. The running conditions were as follows: isocratic 30 mM NaOH (J.T.Baker, NL) for 10 min followed by a linear gradient to 100 mM NaOH for 10 min followed by isocratic 100mM NaOH for 10 min and a linear gradient from 0 to 100 mM Na-acetate (Merck, D) for an additional 35 min. Each run was preceded by a washing and equilibration step: isocratic 500 mM Na-acetate for 5 min followed by isocratic 300 mM NaOH for 10 min followed by isocratic 30mM NaOH for 10 min. The retention times for 6SL and 3SL were 38.3 min and 38.7 min, respectively. Peak identification was done based on retention time comparison with authentic external sialyllactose standards (Dextra laboratories, UK) and disappearance of sialyllactose peaks upon neuraminidase treatment with simultaneous appearance of N-acetylneuraminic acid. For quantification, a standard curve with 50, 100 and 250 ng authentic 6SL and 3Sl standards was established before and after injection of five milk samples.

DSS induced colitis - Seven week old, sex matched mice were treated with 3.75% (w/v) DSS (molecular mass = 36-50 kD; MP Biomedicals, France) in drinking water for 5 days followed by a supply of normal water until sacrifice of the animals (Okayasu I, 1990). The lowest possible DSS dosage was chosen to achieve acute inflammation within 7 days. Body weight and physical activity were monitored daily. Animal pain was kept to a minimum by following the Swiss Animal Protection Ordinance and euthanizing animals reaching less than 85% of initial body weight.

Transepithelial permeability assay - Mice were gavaged with 60 mg/100 g body weight of FITC-dextran (MW 3,000-5,000, Sigma) (Napolitano et al., 1996). Mice were sacrificed and blood was isolated by cardiac puncture. Serum fluorescence (485/535 nm) was measured immediately using a Genios Multi-Detection Microplate Reader (Tecan, Switzerland). Concentrations were calculated from standard curves using serial dilutions of FITC-dextran in serum.

Cytokine gene expression - RNA from frozen colon tissue was isolated using the RNeasy Protect Mini Kit (Qiagen) according to the manufacturer's instructions. RT was performed with 2 µg total RNA using oligo(dT) primers and an Omniscript RT kit (Qiagen, USA). Real-time PCR was carried out using SYBR Green JumpStart Taq ReadyMix (Sigma, Switzerland) with specific primers for IL-1□, IL-6, TNFα and GAPDH (QuantiTec Primer, Qiagen, USA) in a *Mx3000P* thermocycler (Stratagene, USA). Cycling conditions were 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s after initial denaturation at 95°C for 10 min. Gene expression was normalized to GAPDH expression using the $2^{-\Delta\Delta Ct}$ method.

Lamina propria leukocyte isolation - Colon lamina propria leukocytes were isolated as described previously (Lefrançois and Lycke, 2003). Briefly, to detach intraepithelial lymphocytes, chopped colon segments were incubated 2 x 30 min at 37°C under constant stirring condition in 50 ml of Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 10 mM HEPES, 2% horse serum, 2 mM DTT and 0.5 mM EDTA. Leukocytes were released by additional incubation with 0.5 mg/ml of collagenase type IV (Sigma, Switzerland) and 30 µg/ml of DNAse I (Sigma, Switzerland) for 2 x 45 min at 37°C and cells were filtered through a 70 µm nylon mesh cell strainer.

Flow cytometry – Cells were stained on ice for 30 min with anti-CD45 APC-Cy7, anti-CD19 APC, anti-CD3 FITC, anti-Gr.1 PE, anti-TCRγδ PE, anti-TCRβ APC, anti-CD4 PE, anti-CD8β PE and anti-CD8α APC antibodies (BD Biosciences, USA) and analyzed with a FACSCanto II flow cytometer (BD Biosciences, USA).

Bone marrow transfer - Ten week old male Wt and *St3gal4*-null recipient mice were lethally exposed to 9.33 Gy radiations (3.11 Gy/min). Femur and tibia from Wt and *St3gal4*-null donor mice were removed and flushed with RPMI 10% FCS (GIBCO, USA) to harvest BM cells. Irradiated recipient mice were reconstituted with 2 x 10^6 BM cells by intravenous injection (Spangrude, 2008). Mice were treated with antibiotics (Borgal® 24 % ad us. vet.,Veterinaria AG, Zürich, Switzerland) for 3 weeks.

Temporal temperature gradient gel electrophoresis – DNA was isolated from freshly isolated cecal content using the QIAamp DNA Stool Mini Kit (Qiagen, USA). Bacterial 16S rRNA DNA was amplified using the universal primers HDA1-GC 5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGG-GGGGACTCCTACGGGAGGCAGCAGT-3' and HDA-2 5'-GTATTACCGCGGCTGCTGGCAC-3' according to Ogier (Ogier et al., 2002). The PCR conditions were 30 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min flanked by an initial denaturation at 94°C for 4 min and a final elongation at 72°C for 4 min. PCR products were loaded on 8.5% polyacrylamide gels containing 8 M urea and separated by temperature gradient gel electrophoresis using the D-Code universal mutation detection system (Bio-Rad, USA). Electrophoresis was performed in 60 mM Tris-acetate, 30 mM acetic acid and 1.5 mM EDTA first at 20 V for 15 min followed by at constant voltage of 80 V for 18 hours with a temperature increase of 0.2°C/h from 66 to 70°C. TTGE profiles were analyzed by using GelCompar II software (Applied Maths NV, Belgium).

Bacterial typisation –TTGE bands were excised from the gels and DNA was diffused over night at 4°C in 100 µl H_2O. Standard HDA primers were used to reamplify the PCR product from 3 µl

of eluate. PCR products were purified using QIAquick PCR purification kit (Qiagen) and sequenced (Synergene Biotech, Switzerland). The proportion of bacterial phyla in intestinal samples was determined by real-time PCR using the SYBR Green JumpStart Taq ReadyMix (Sigma) as described for cytokine analysis here above and primer pairs specific for the lineages *Bacteroidetes, Enterobacteriaceae, Lachnospiraceae,* clostridial cluster IV and *Lactobacillaceae* (table1) The forward primer specific for the *Ruminococcaceae* species was designed by choosing a stretch of 16S RNA gene sequence that was distinctive from the corresponding sequences of other *Ruminococcaceae* of the clostridial cluster IV. Cycling conditions were 40 cycles at 95°C for 15 s, 66°C for 20 s and 72°C for 20 s after an initial denaturation at 95°C for 10 min. Quantification values were calculated by the $2^{-\Delta Ct}$ method relative to total bacteria 16S rDNA amplification.

Germfree colonization - Cecal contents (100 mg) of Wt and *St3gal4*-null donor mice were collected under anaerobic conditions and diluted in 10 ml of anaerobic mineral solution containing 5 g/l NaCl, 2 g/l glucose, and 0.3 g/l cysteine-HCl (de Sablet et al., 2009). Three to four week old C57Bl/6 germfree males (Institute of Laboratory Animal Science, University of Zürich, Switzerland) were colonized with 200 µl of 1:100-diluted cecal microbiota by gavage and kept in isolators for 4 weeks.

Statistics - Results were expressed as mean ± SEM. Difference between groups was analyzed using one-way ANOVA with Bonferroni's Multiple Comparison Post-test. Significance was accepted for $p<0.05$.

Acknowledgements

The authors kindly thank Monique Julita and John Newell for excellent technical assistance with milk oligosaccharide and mammary gland gene expression analysis. This work was supported by the Zürich Center for Integrative Human Physiology and by the Swiss National Foundation grant 31003A-116039 to TH.

REFERENCES

Almagro-Moreno, S., and E.F. Boyd. 2009a. Insights into the evolution of sialic acid catabolism among bacteria. *BMC Evol Biol.* 9:118.

Almagro-Moreno, S., and E.F. Boyd. 2009b. Sialic acid catabolism confers a competitive advantage to pathogenic vibrio cholerae in the mouse intestine. *Infect Immun.* 77:3807-3816.

Andoh, A., S. Sakata, Y. Koizumi, K. Mitsuyama, Y. Fujiyama, and Y. Benno. 2007. Terminal restriction fragment length polymorphism analysis of the diversity of fecal microbiota in patients with ulcerative colitis. *Inflamm Bowel Dis.* 13:955-962.

Bartosch, S., A. Fite, G.T. Macfarlane, and M.E.T. McMurdo. 2004. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl Environ Microbiol.* 70:3575-3581.

Bode, L. 2009. Human milk oligosaccharides: prebiotics and beyond. *Nutr Rev.* 67:S183-S191.

Brand Miller, J., P. McVeagh, Y. McNeil, and B. Gillard. 1995. Human milk oligosaccharides are not digested and absorbed in the small intestine of young infants. *Proc Nutr Soc Aus.* 19:44.

Chang, D.-E., D.J. Smalley, D.L. Tucker, M.P. Leatham, W.E. Norris, S.J. Stevenson, A.B. Anderson, J.E. Grissom, D.C. Laux, P.S. Cohen, and T. Conway. 2004. Carbon nutrition of Escherichia coli in the mouse intestine. *Proc Natl Acad Sci U S A* 101:7427-7432.

Collins, M.D., P.A. Lawson, A. Willems, J.J. Cordoba, J. Fernandez-garayzabal, P. Garcia, J. Cai, H. Hippe, and J.A.E. Farrow. 1994. The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* 44:812-826.

Dalziel, M., R.Y. Huang, F. Dall'Olio, J.R. Morris, J. Taylor-Papadimitriou, and J.T.Y. Lau. 2001. Mouse ST6Gal sialyltransferase gene expression during mammary gland lactation. *Glycobiology* 11:407-412.

de Sablet, T., C. Chassard, A. Bernalier-Donadille, M. Vareille, A.P. Gobert, and C. Martin. 2009. Human microbiota-secreted factors inhibit shiga toxin synthesis by enterohemorrhagic Escherichia coli O157:H7. *Infect Immun.* 77:783-790.

Dethlefsen, L., M. McFall-Ngai, and D.A. Relman. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* 449:811-818.

Eckburg, P.B., E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S.R. Gill, K.E. Nelson, and D.A. Relman. 2005. Diversity of the human intestinal microbial flora. *Science* 308:1635-1638.

Egge, H. 1993. The diversity of oligosaccharides in human milk. *In New Perspectives in Infant Nutrition* ed. B Renner, G Sawatzki:pp 12-26.

Ellies, L.G., D. Ditto, G.G. Levy, M. Wahrenbrock, D. Ginsburg, A. Varki, D.T. Le, and J.D. Marth. 2002. Sialyltransferase ST3Gal-IV operates as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor ligands. *Proc Natl Acad Sci U S A* 99:10042-10047.

Falk, P.G., L.V. Hooper, T. Midtvedt, and J. Gordon. 1998. Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol Mol Biol Rev..* 62:1157-1170.

Frank, D.N., and N.R. Pace. 2008. Gastrointestinal microbiology enters the metagenomics era. *Curr Opin Gastroenterol.* 24:4-10.

Frank, D.N., A.L. St. Amand, R.A. Feldman, E.C. Boedeker, N. Harpaz, and N.R. Pace. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A* 104:13780-13785.

Furet, J.-P., O. Firmesse, M. Gourmelon, C. Bridonneau, J. Tap, S. Mondot, J. Doré, and G. Corthier. 2009. Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. *FEMS Microbiol Ecol.* 68:351-362.

Gaboriau-Routhiau, V., S. Rakotobe, E. Lécuyer, I. Mulder, A. Lan, C. Bridonneau, V. Rochet, A. Pisi, M. De Paepe, G. Brandi, G. Eberl, J. Snel, D. Kelly, and N. Cerf-Bensussan. 2009. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity* 31:677-689.

Gibson, G.R., and M.B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr.* 125:1401-1412.

Haberman, B. 1974. Mechanical milk collection from mice for Bittner virus isolation. *Lab Anim Sci* 6:935-937.

Harduin-Lepers, A., V. Vallejo-Ruiz, M.A. Krzewinski-Recchi, B. Samyn-Petit, S. Julien, and P. Delannoy. 2001. The human sialyltransferase family. *BMC Evol Biol.* 83:727-737.

Harmsen, H.J.M., A.C.M. Wildeboer-Veloo, G.C. Raangs, A.A. Wagendorp, N. Klijn, J.G. Bindels, and G.W. Welling. 2000. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr.* 30:61-67.

Hennet, T., D. Chui, J.C. Paulson, and J.D. Marth. 1998. Immune regulation by the ST6Gal sialyltransferase. *Proc Natl Acad Sci U S A.* 95:4504-4509.

Herbeck, J.L., and M.P. Bryant. 1974. Nutritional Features of the Intestinal Anaerobe *Ruminococcus bromii*. *Appl Microbiol.* 28:1018-1022.

Hoffen, E.v., B. Ruiter, J. Faber, L. M'Rabet, E.F. Knol, B. Stahl, S. Arslanoglu, G. Moro, G. Boehm, and J. Garssen. 2009. A specific mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides induces a beneficial immunoglobulin profile in infants at high risk for allergy. *Allergy* 64:484-487.

Im, E., Y.J. Choi, C. Pothoulakis, and S.H. Rhee. 2009. Bacillus polyfermenticus Ameliorates Colonic Inflammation by Promoting Cytoprotective Effects in Colitic Mice. *J Nutr.* 139:1848-1854.

Kuhn, J.N. 1972. The lactose and neuraminlactose content of rat milk and mammary tissue. *Biochem J.* 130:177-180.

Kumar, N.S.N., R. Balamurugan, K. Jayakanthan, A. Pulimood, S. Pugazhendhi, and B.S. Ramakrishna. 2008. Probiotic administration alters the gut flora and attenuates colitis in mice administered dextran sodium sulfate. *J Gastroenterol Hepatol.* 23:1834-1839.

Lefrançois, L., and N. Lycke. 2003. Isolation of mouse small intestinal intraepithelial lymphocytes, Peyer's patch, and lamina propria cells. *Curr Protoc Immunol.* Chapter3:Unit 3.19.

Leitch, E.C.M., A.W. Walker, S.H. Duncan, G. Holtrop, and H.J. Flint. 2007. Selective colonization of insoluble substrates by human faecal bacteria. *Environ Microbiol.* 9:667-679.

Ley, R.E., D.A. Peterson, and J.I. Gordon. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124:837-848.

Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-408.

Macpherson, A.J., and N.L. Harris. 2004. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* 4:478-485.

Martinez-Medina, M., X. Aldeguer, F. Gonzalez-Huix, D. Acero, and L.J. Garcia-Gil. 2006. Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis. *Inflamm Bowel Dis.* 12:1136-1145.

Moro, G., S. Arslanoglu, B. Stahl, J. Jelinek, U. Wahn, and G. Boehm. 2006. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. *Arch Dis Child.* 91:814-819.

Nagasawa, H. 1979. A device for milk collection from mice. *Lab Anim Sci* 5:633-635.

Napolitano, L., M. Koruda, A. Meyer, and C. Baker. 1996. The impact of femur fracture with associated soft tissue injury on immune function and intestinal permeability. *Shock* 5:202-207.

Newburg, D.S. 2009. Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. *J Anim Sci.* 87:26-34.

Ogier, J.-C., O. Son, A. Gruss, P. Tailliez, and A. Delacroix-Buchet. 2002. Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. *Appl Environ Microbiol.* 68:3691-3701.

Okayasu I, H.S., Yamada M, Ohkusa T, Inagaki Y, Nakaya R. 1990. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 98:694-702.

Pirindiville, T., M. Centrell, and K. Wilson. 2004. Ribosomal DNA sequence analysis of mucosa-associated bacteria in Crohn's disease. *Inflamm Bowel Dis.* 10:824-833.

Priatel, J.J., D. Chui, N. Hiraoka, C.J. Simmons, K.B. Richardson, D.M. Page, M. Fukuda, and J.D. Marth. 2000. The ST3Gal-I sialyltransferase controls $CD8^+$ T lymphocyte homeostasis by modulating O-glycan biosynthesis. *Immunity* 12:273-283.

Prieto, P.A., P. Mukerji, B. Kelder, R. Erney, D. Gonzalez, J.S. Yun, D.F. Smith, K.W. Moremen, C. Nardelli, M. Pierce, Y. Li, X. Chen, T.E. Wagner, R.D. Cummings, and J.J. Kopchick. 1995. Remodeling of mouse milk glycoconjugates by transgenic expression of a human glycosyltransferase. *J Biol Chem.* 270:29515-29519.

Ramirez-Farias, C., K. Slezak, Z. Fuller, A. Duncan, G. Holtrop, and P. Louis. 2009. Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii. *Br J Nutr.* 101:541-550.

Rinttilä, T., A. Kassinen, E. Malinen, L. Krogius, and A. Palva. 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 97:1166-1177.

Sakarya, S., G.T. Ertem, S. Oncu, I. Kocak, N. Erol, and S. Oncu. 2003. *Escherichia coli* bind to urinary bladder epithelium through nonspecific sialic acid mediated adherence. *FEMS Immunol Med Microbiol.* 39:45-50.

Savage, D.C. 1977. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol.* 31:107-133.

Sohanpal, B.K., S. El-Labany, M. Lahooti, J.A. Plumbridge, and I.C. Blomfield. 2004. Integrated regulatory responses of fimB to N-acetylneuraminic (sialic) acid and GlcNAc in Escherichia coli K-12. *Proc Natl Acad Sci U S A.* 101:16322-16327.

Spangrude, G.J. 2008. Assessment of lymphocyte development in radiation bone marrow chimeras. *Curr Protoc Immunol.* Chapter 4:Unit 4.6.

Turnbaugh, P.J., R.E. Ley, M.A. Mahowald, V. Magrini, E.R. Mardis, and J.I. Gordon. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444:1027-1131.

Urashima, T., T. Saito, T. Nakamura, and M. Messer. 2001. Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconj J.* 18:357-371.

Van Dyke, M.I., and A.J. McCarthy. 2002. Molecular biological detection and characterization of Clostridium populations in municipal landfill sites. *Appl Environ Microbiol.* 68:2049-2053.

Vijay-Kumar, M., J.D. Aitken, F.A. Carvalho, T.C. Cullender, S. Mwangi, S. Srinivasan, S.V. Sitaraman, R. Knight, R.E. Ley, and A.T. Gewirtz. 2010. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. *Science* 328:228-231.

Vos, A.P., M. Haarman, J.-W.H. vanGinkel, J. Knol, J. Garssen, B. Stahl, G. Boehm, and L. M'Rabet. 2007. Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice. *Pediatr Allergy Immunol.* 18:304-312.

Walter, J., G.W. Tannock, A. Tilsala-Timisjarvi, S. Rodtong, D.M. Loach, K. Munro, and T. Alatossava. 2000. Detection and identification of gastrointestinal Lactobacillus species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl Environ Microbiol.* 66:297-303.

Wang, R.F., W.W. Cao, and C.E. Cerniglia. 1997. PCR detection of Ruminococcusspp. in human and animal faecal samples. *Mol Cell Probes.* 11:259-265.

Figure legends

Figure 1. Sialyltransferase gene expression in mouse mammary gland. The relative mRNA levels of the six α2,3 sialyltransferases *St3gal1* to *St3gal6* and of the two α2,6 sialyltransferases *St6gal1* and *St6gal2* was measured in virgin mice (mRNA levels set to 1) at 1 week postpartum, 2 weeks postpartum and 3 days post weaning (N = 3).

Figure 2. Sialyllactose concentration in mouse milk during lactation. (A) Sialyllactose in milk from Wt, *St3gal1*-, *St3gal4*-, *St6gal1*-null mice isolated at day 2 postpartum. α2,3- (3SL, solid line) and α2,6- (6SL, dashed line) linked sialyllactose were measured in Wt milk **(B)** and in *St3gal4*-null milk **(C)** throughout lactation. Amounts are given in g per 100 g dried milk. Values are measured by mean ± SEM (N = 3).

Figure 3. DSS-induced colitis. (A) Body weight was determined daily in Wt mice (● solid line), cross-fostered Wt mice (▲ dashed line), *St3gal4*-null mice (● solid line) and cross-fostered *St3gal4*-null mice (▲ dashed line). **(B)** Reduction of colon length. Values are given as percentage of untreated control mice; C, control mice, DSS, DSS-treated mice, CF/DSS, cross-fostered DSS-treated mice. **(C)** Intestinal permeability as measured by FITC-dextran levels in serum from controls and colitogenic mice on day 5 of DSS induced colitis (N = 4-8). **(D-F)** Cytokine gene expression at day 5 of DSS treatment. Measurements were performed using $2^{-\Delta\Delta ct}$ method with GAPDH as housekeeping gene (N = 8). Data are represented by mean ± SEM, * $p<0.05$.

Figure 4. Hematopoietic cell infiltration and bone marrow chimera of Wt and *St3gal4*-null mice. Colonic cells were isolated and stained with anti-CD45 APC-Cy7 antibody and measured by FACS. Mice were analyzed on day 0, 5 and 7 of DSS treatment. **(A)** Representative dot plots of Wt, cross-fostered Wt mice (Wt CF), *St3gal4*-null and cross-fostered *St3gal4*-null mice (St3gal4-null CF). **(B)** Quantitation of $CD45^+$ cells in colon infiltrates (N = 4-8). **(C)** Body weight of Wt mice transplanted with *St3gal4*-null bone marrow cells (▲, N = 10) and *St3gal4*-null mice transplanted Wt bone marrow cells (●, N = 11) during DSS treatment. Error bars are expressed as mean ± SEM, * $p<0.05$.

Figure 5. Microbiota analysis in the mouse gastrointestinal tract. TTGE profiles of 16S rDNA amplification products from cecum of 6 week-old Wt and *St3gal4*-null mice fed with either Wt or *St3gal4*-null milk **(A)** and after DSS induced colitis **(B)**. The marker (M) shows amplification products from the species *Lactobacillus plantarum, Lactococcus lactis, Corynebacterium variabile, Brevibacterium linens, Arthrobacter protophormiae* from top to bottom. Arrows (►) mark the position corresponding to the *Ruminococcaceae* bands (N = 6).

Figure 6. Quantitative analysis of gastrointestinal microbiota. The microbiota composition of Wt, *St3gal4*-null and respective cross-fostered (CF) mice was determined by real-time PCR in **(A)** native microbiota, and **(B)** microbiota on day 7 of DSS treatment. **(C)** Real-time PCR of *Ruminococcaceae* species in Wt, *St3gal4*-null and CF mice in controls and after 7 days of DSS induced colitis. Values are shown as relative amount to total bacteria 16S rDNA measured by the $2^{-\Delta Ct}$ method. Error bars are expressed as mean ± SEM (N = 4-6), * $p<0.05$.

Figure 7. Colitis in reconstituted germfree mice. **(A)** TTGE profiles of 16S rDNA amplification products from feces of mice colonized with Wt or *St3gal4*-null microbiota at two weeks post colonization (N = 6). **(B)** Body weight change in DSS-induced colitis of reconstituted germfree mice colonized with Wt (▼) or *St3gal4*-null (♦) microbiota (N = 9-11). The difference in body weights measured at day 7 was significant ($p<0.05$)

Table I: List of primers for the amplification of sialyltransferase genes and bacterial populations

Names	Primers	Sequences 3'-5'	References
St3Gal1	mQ_S3G-I Fwd	CACCTGCAGACACTGCATCAG	(present study)
	mQ_S3G-I Rev	TCAGAGCGTTGTGGACTGTCA	
St3Gal2	mQ_S3G-II Fwd	CCGAACAACTCACCATTTCATG	(present study)
	mQ_S3G-II Rev	GCGCTGGCAATCCACATTA	
St3Gal3	mQ_S3G-III Fwd	GGCTACGCTTCAGCCATGA	(present study)
	mQ_S3G-III Rev	CCCAAAAGGTGGCACAAACT	
St3Gal4	mQ_S3G-IV Fwd	TCCAGGGTGAGGCAGAGAGA	(present study)
	mQ_S3G-IV Rev	GTCCCAAAGGGCAGCTCATA	
St3Gal5	mQ_S3G-V Fwd	GCAGCCTCCACAGGGACTT	(present study)
	mQ_S3G-V Rev	AATAGGACGAAGAGGTGCACAGA	
St3Gal6	mQ_S3G-VI Fwd	CCCAAGGATCAGAAACCCAAA	(present study)
	mQ_S3G-VI Rev	CATGGTGGCATTCCCGTAGT	
St6Gal1	mQ_S6G-I Fwd	GCGCAAGACAGATGTGTGCTA	(present study)
	mQ_S6G-I Rev	TCTGTTCCCTCATTGAGATGCTT	
St6Gal2	mQ_S6G-II Fwd	CTATGACAGCGACATGCTGTCA	(present study)
	mQ_S6G-II Rev	CACCATGCTTGTTGAAGGACAT	
Total Bacteria	515F	GTGCCAGCMGCCGCGGTAA	(Frank et al., 2007)
	805R	GACTACCAGGGTATCTAAT	
Bacteroidetes	Bac303R	CCAATGTGGGGGACCTTC	(Frank et al., 2007)
	Bac32F	AACGCTAGCTACAGGCTT	
Enterobacteriaceae	Eco1457F	CATTGACGTTACCCGCAGAAGAAGC	(Bartosch et al., 2004)

	Eco1652R	CTCTACGAGACTCAAGCTTGC	
Lachnospiraceae	Ccocc1F	CGGTACCTGACTAAGAAGC	(Rinttilä et al., 2004)
	Ccocc1R	AGTTTYATTCTTGCGAACG	
Clostridial cluster IV	Clep866mF	TTAACACAATAAGTWATCCACCTGG	(Ramirez-Farias et al., 2009)
	Clept1240mR	ACCTTCCTCCGTTTTGTCAAC	
Lactobacillaceae	F_Lacto 05	AGCAGTAGGGAATCTTCC	(Furet et al., 2009)
	AR_Lacto 04	CGCCACTGGTGTTCYTCCATATA	
Ruminococcaceae species	*Ruminococcaceae*	CTAGGTGAAGATACTGACGGTAACCTG	(present study)
	HDA-2	GTATTACCGCGGCTGCTGGCAC	(Walter et al., 2000)

Figure 1

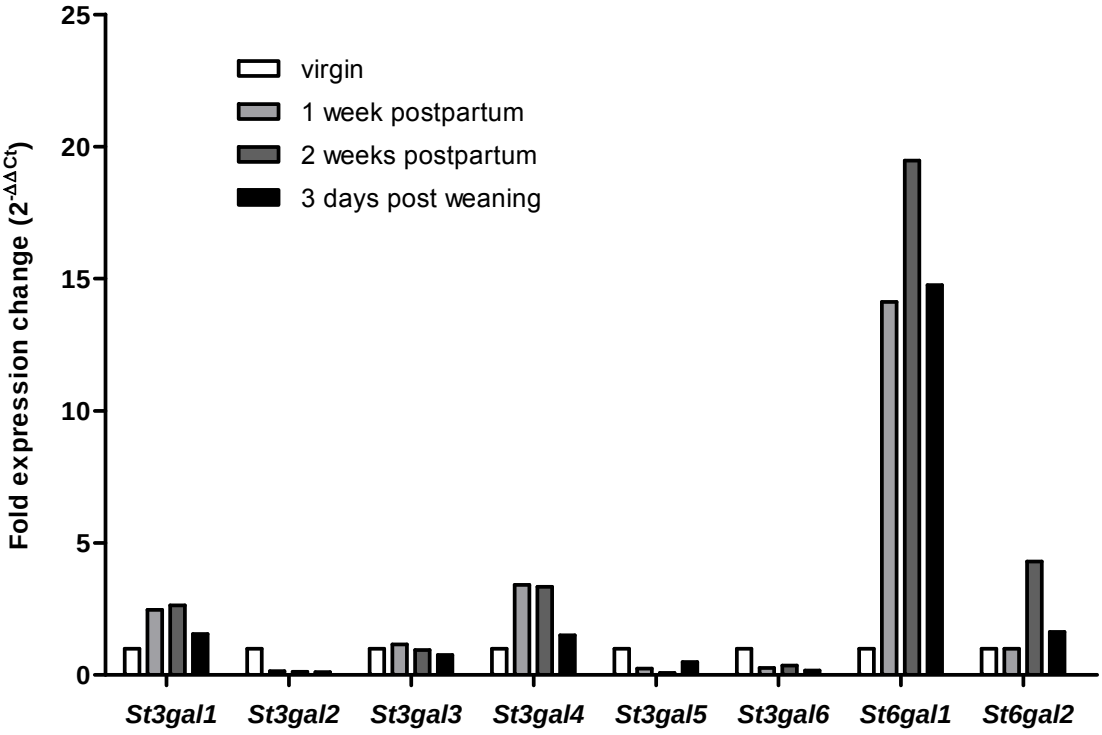

25
20
15
10
5
0
Fold expression change ($2^{-\Delta\Delta Ct}$)
virgin
1 week postpartum
2 weeks postpartum
3 days post weaning
St3gal1
St3gal2
St3gal3
St3gal4
St3gal5
St3gal6
St6gal1
St6gal2

Figure 2

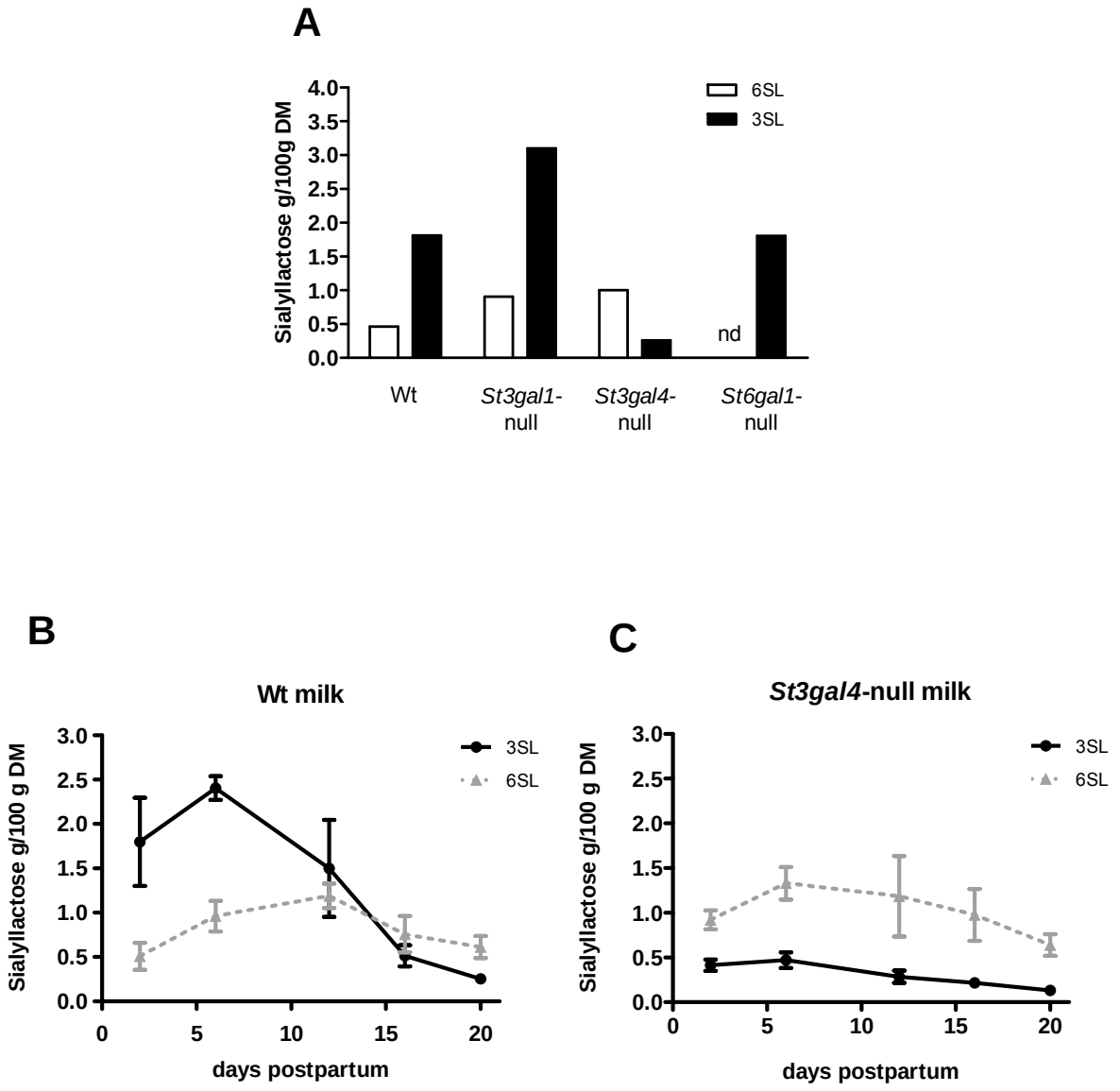

A
Sialyllactose g/100g DM
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
6SL
3SL
nd
Wt
St3gal1-null
St3gal4-null
St6gal1-null
B
Wt milk
Sialyllactose g/100 g DM
3SL
6SL
days postpartum
C
St3gal4-null milk
Sialyllactose g/100 g DM
3SL
6SL
days postpartum

Figure 3

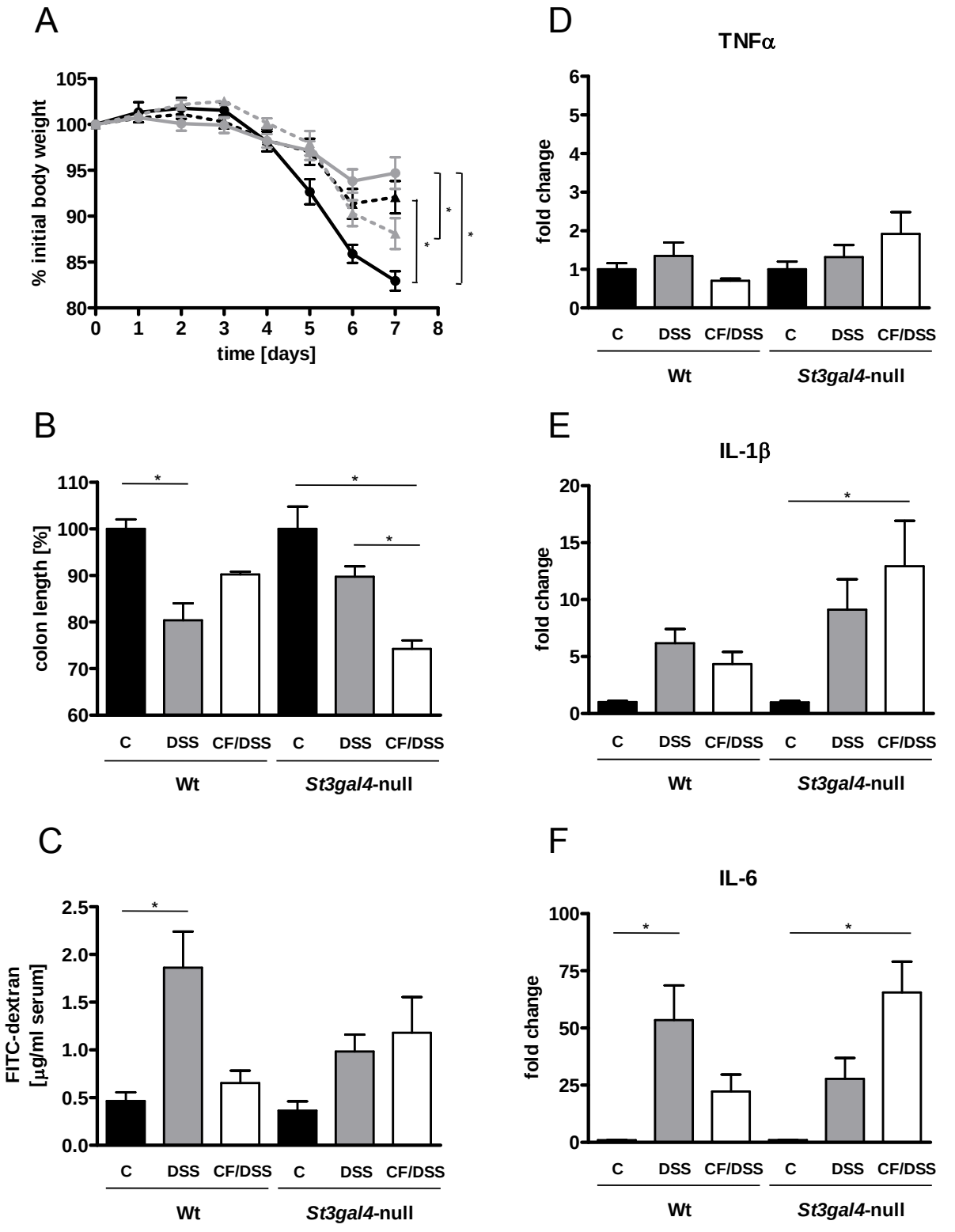

A
% initial body weight
time [days]
B
colon length [%]
C
FITC-dextran [µg/ml serum]
D
TNFα
fold change
E
IL-1β
fold change
F
IL-6
fold change
C
DSS
CF/DSS
Wt
St3gal4-null

Figure 4

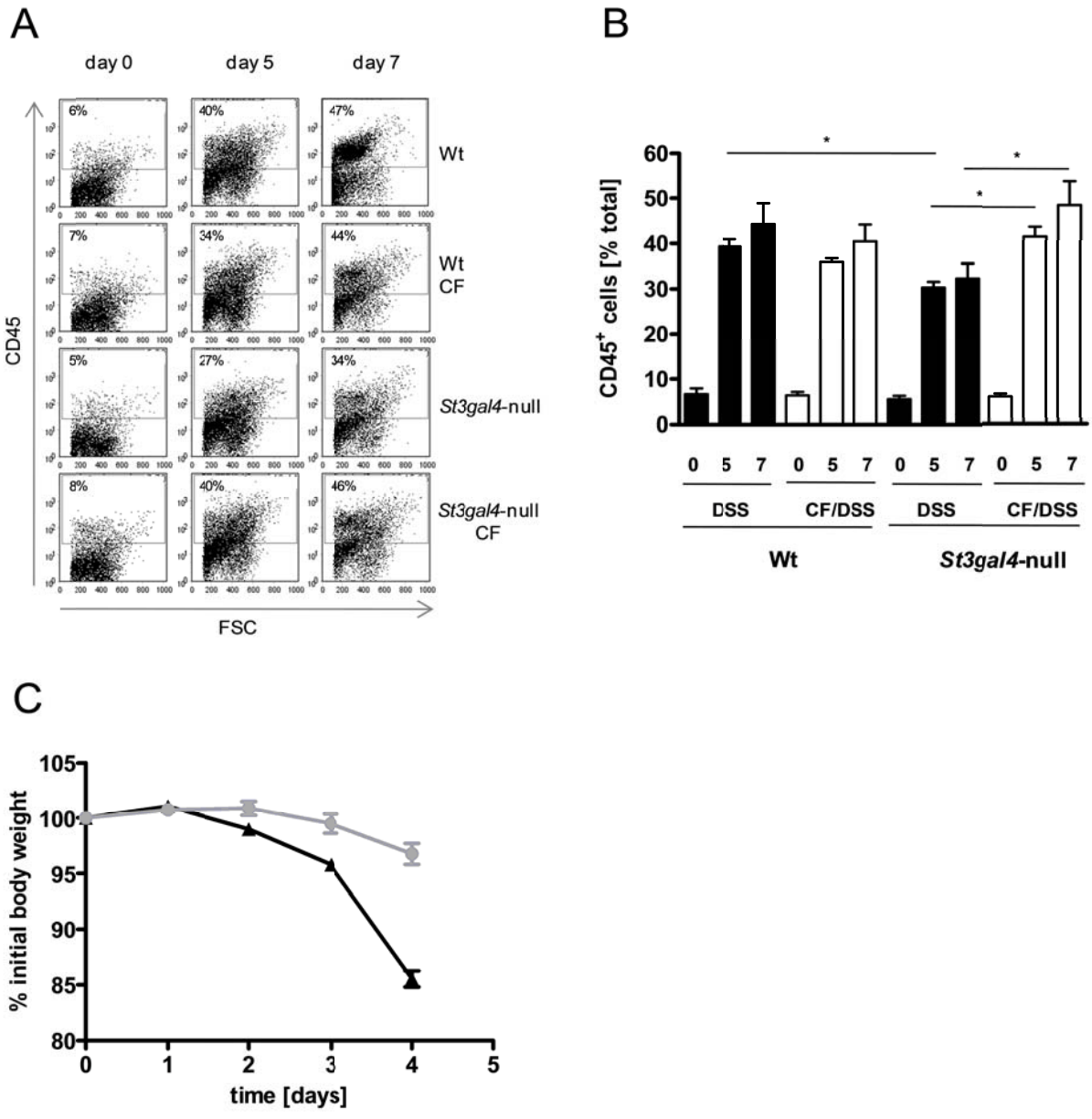

A
day 0
day 5
day 7
6%
40%
47%
Wt
7%
34%
44%
Wt
CF
5%
27%
34%
St3gal4-null
8%
40%
46%
St3gal4-null
CF
CD45
FSC
B
CD45+ cells [% total]
0
10
20
30
40
50
60
0 5 7 0 5 7 0 5 7 0 5 7
DSS
CF/DSS
DSS
CF/DSS
Wt
St3gal4-null
C
% initial body weight
105
100
95
90
85
80
0 1 2 3 4 5
time [days]

Figure 5

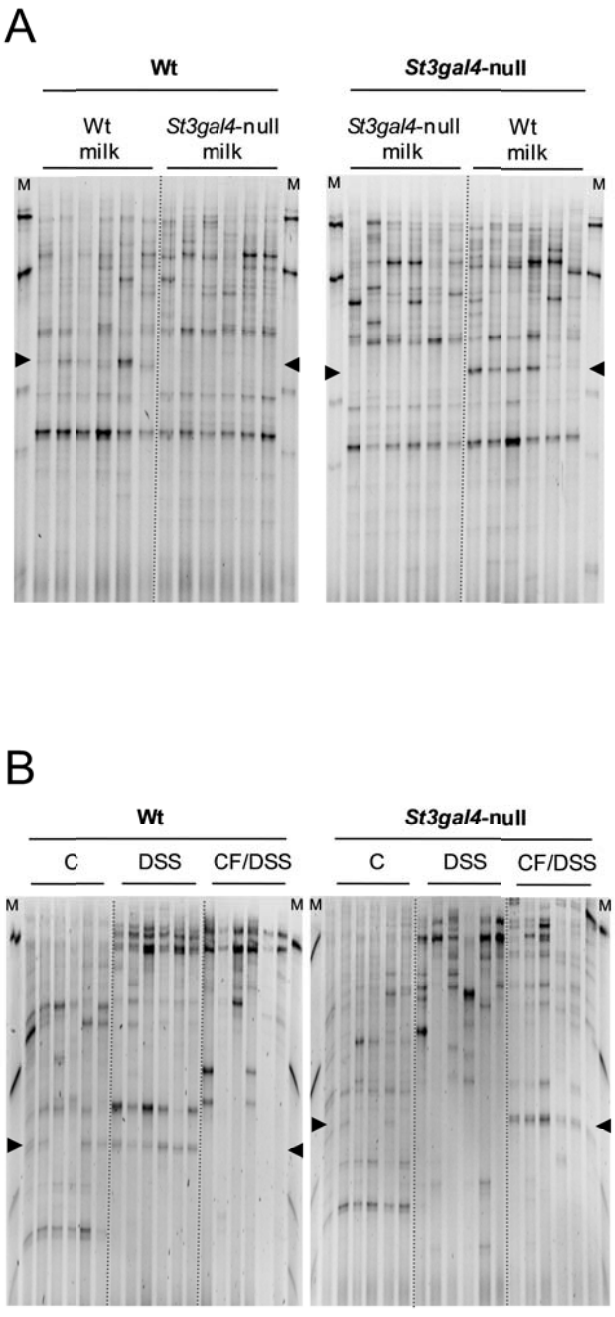

A
Wt
St3gal4-null
Wt milk
St3gal4-null milk
St3gal4-null milk
Wt milk
M
M
M
M
B
Wt
St3gal4-null
C
DSS
CF/DSS
C
DSS
CF/DSS
M
M
M
M

Figure 6

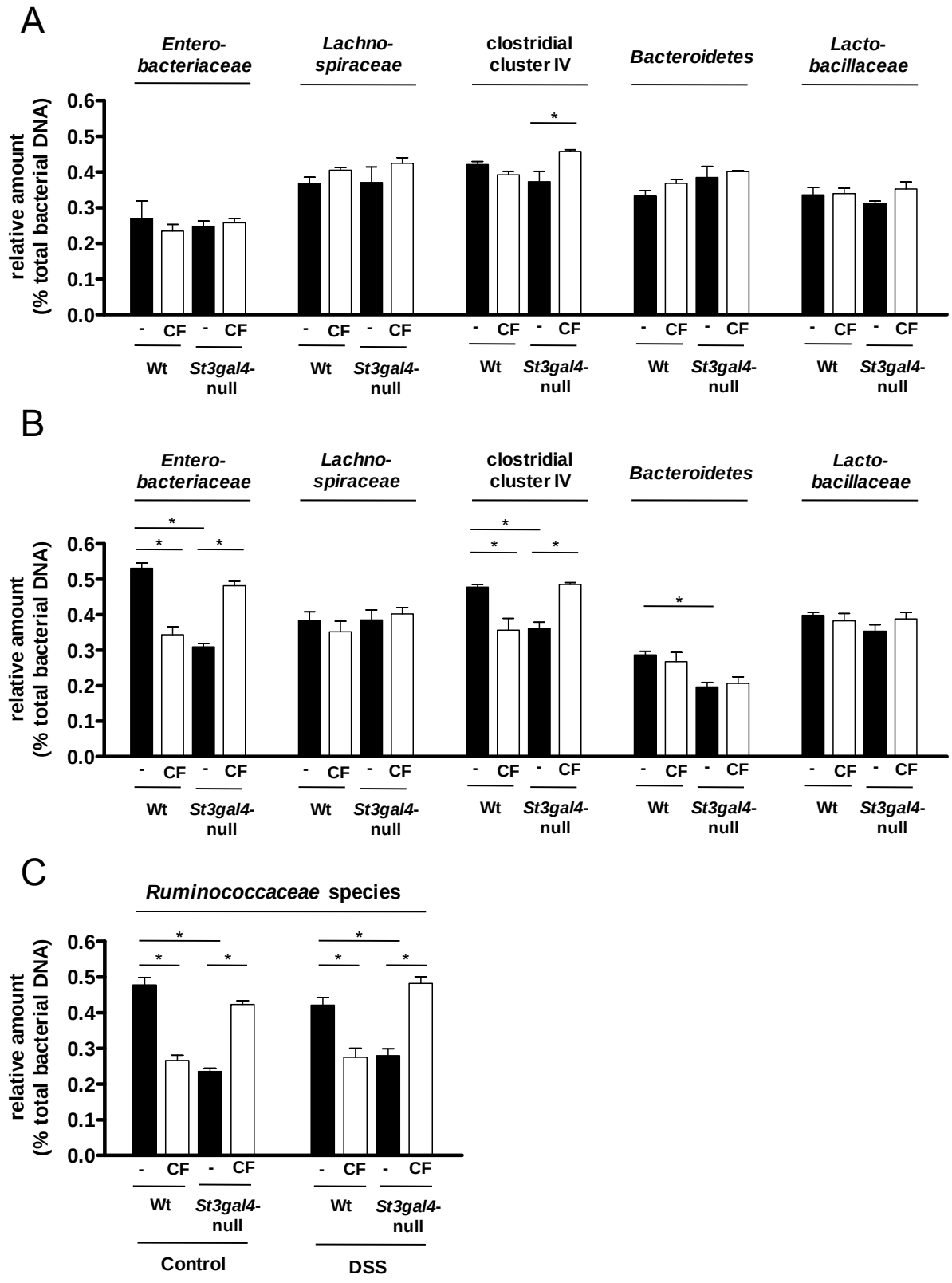
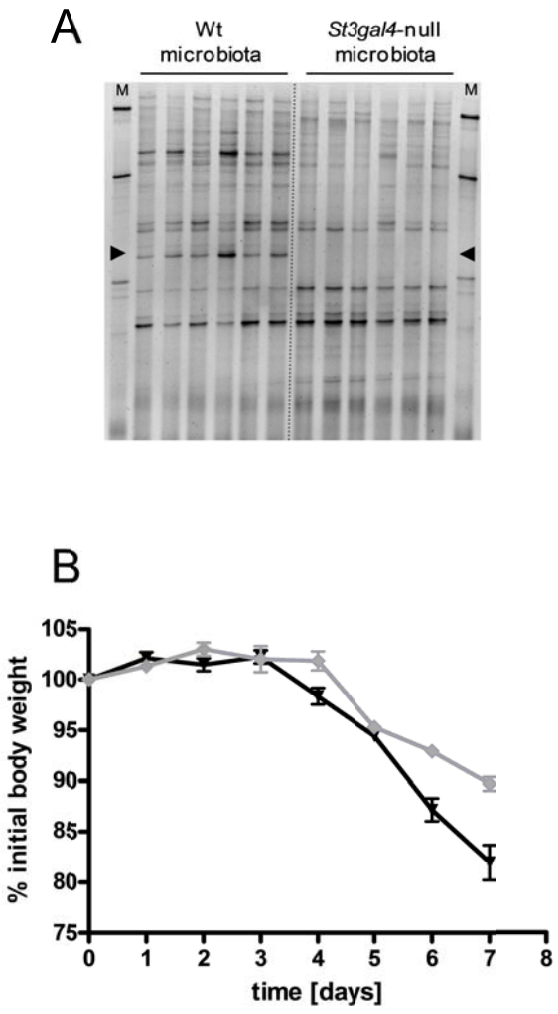

A
Entero-bacteriaceae
Lachno-spiraceae
clostridial cluster IV
Bacteroidetes
Lacto-bacillaceae
relative amount (% total bacterial DNA)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
- CF - CF
Wt
St3gal4-null
B
Entero-bacteriaceae
Lachno-spiraceae
clostridial cluster IV
Bacteroidetes
Lacto-bacillaceae
relative amount (% total bacterial DNA)
*
- CF - CF
Wt
St3gal4-null
C
Ruminococcaceae species
relative amount (% total bacterial DNA)
- CF - CF
Wt
St3gal4-null
Control
DSS

Figure 7


A
Wt microbiota
St3gal4-null microbiota
M
M
B
% initial body weight
105
100
95
90
85
80
75
0
1
2
3
4
5
6
7
8
time [days]

Discussion and future directions

References

Acknowledgments

I would like to start my acknowledgments by thanking **all** the people who accompanied me during my PhD studies. Without you it would not have been possible.

First of all I would like to thank **Prof. Dr. Thierry Hennet** for giving me the possibility to work in his lab on such an exciting project. I am grateful not only for his scientific inspiration but also for sharing his tips and tricks in improving my skills in photography.

Further I would like to acknowledge my committee members **Prof. Dr. med. Eric G. Berger**, **Prof. Dr. Thomas Brunner** and **Dr. Norbert Sprenger** for their contribution to the success of this thesis. I enjoyed the conversation with Eric in REAL Swiss-German which helped me feel less homesick in the "big city". I thank Thomi for introducing me to the most fascinating research field (mucosal immunology) and for still inviting me to the nice apéros in Bern. Further I thank Norbert for the fruitful collaboration and of course for the wonderful "X-mas Schoggi" that our lab enjoyed each December.

Special thanks to **Dr. Lubor Borsig** for his help in performing animal experiments especially when trying i.v. injections and retro-orbital bleeding in mice. **Christoph Rutschmann**, the good soul of the lab, for never loosing patients with us girls in the L80. Then, very special thanks to my close friends **Dr. Belinda Schegg** and **Dr. Charlotte Maag** who gave me scientific and moral support during my PhD. Thank you Belinda for being such a great bench and office neighbor and for the good laughs we had while doing crazy things. Thank you Charlotte for keeping up my sports activities and for the nice lunch breaks down at the "lake". Thanks go also to **Dr. Franziska Biellmann** for showing me the difference between female and male mice and for her support while writing my thesis. Big thanks to **Dr. Kelvin Luther** for taking the time correcting my thesis, it was extremely helpful (genau, genau…). For taking care of my animals and for his friendly smile every time I entered the mouse facility I thank **Matthew Adjei**.

Then sincere thanks to all the current and former lab members: **Jür Cabalzar** (viva la Grischa), **Dr. Stefan Deuber**, **Micha Häuptle**, **Dr. Andreas Hülsmeier**, **Esther Quinziano, Dr. Albana Rexhepaj, Daniel Rhyner**, **Giovanna Roth** (the best secretary ever), **Nikunj Shah** (Chapati with basic knowledge in German), **Dr. Claire Tricaud-Perrin**, **Katja Trompf** (Sushi-queen), **Gisela Adrienne Weiss**, **Michael Welti** (master of Ju-Jitsu). I also thank all the members from the Borsig, Rohrer and Wüthrich group for creating a wonderful working atmosphere on the L floor. Additionally to the L-floor people I would like to thank **Paul Heiniger**, **Heidi Preisig**, **Philippe Schläfli** and **Patrick Spielmann** for the nice coffee breaks and after work beers.

I would also like to thank a few people outside the Institute of Physiology that contributed to the success of my thesis. I specially thank **Dr. Christophe Chassard** for introducing me into the field of gut microbiology and **Dr. Emmanuelle Roth** for her help with the TTGE analysis. I thank **Leo Mamaril** and **Ruedi Jörg** from the LTK for providing and helping me with the germfree mice.

Special thanks go to three real great friends that I made during my four years at the University of Zurich, **Dr. Astrid Starke**, **Nicola Schäfer** and **Stefanie Schanz Jurinka**. I thank Astrid for surviving the LTK course with me, for technical and scientific support and for always being there for me. I thank Nicola for our spontaneous “Frust Bier” any time of the day, for being my US travel partner, for the good laughs and for being a great friend. I thank Steffi for her support and encouragement even after leaving the lab. You are all great!

The past few years would have not been possible without you: **Susanne Szentkuti**, **Daniela Kassahn**, **Sybille Matthey**, **Sonja und Aris Neidhart**, **Corinne Schär** (travel partner and colitis specialist), **Céline Bürki**.

Last but not least, I express my deepest gratitude to my family **Bernard**, **Marc**, **Reto** and **Ursula Fuhrer**. I especially thank my mom for all she has done for me in good and bad times, I am very grateful!

Curriculum Vitae

Name	Andrea Corinne **FUHRER**
Date of birth	24.01.1980
Citizen of	Trubschachen BE

Education

2006-2010	PhD thesis at the Institute of Physiology, University of Zürich, in the group of Prof. Dr. Thierry Hennet "Regulatory Functions of Sialylated Milk Oligosaccharides in Mucosal Immunity"
2004-2005	Diploma thesis at the Institute of Pathology, University of Bern, in the group of Prof. Dr. Thomas Brunner "Targeted Inactivation of the Glucocorticoid Synthesis in the Intestinal Mucosa"
2003-2004	Erasmus/Socrates semester at the Université Montpellier II (Sciences et Techniques du Languedoc)
2000-2005	Studies in Biology at the University of Bern with major in cell biology
1995-2000	High school education at the Gymnasium Bern Neufeld, graduated in economics (Typus E)

Publications

Cima I, **Fuhrer A**, and Brunner T. 2006. "Antagonistic and synergistic effects of glucocorticoids and IL-7 on $CD4^+$ T cell activation." Immunology Letters 106(1): 99-102.

Mueller M, Cima I, Noti M, Fuhrer A, Jakob S, Dubuquoy L, Schoonjans K, and Brunner T. 2006. "The nuclear receptor LRH-1 critically regulates extra-adrenal glucocorticoid synthesis in the intestine." J. Exp. Med. 203(9): 2057-2062.

Cima I, Corazza N, Dick B, **Fuhrer A**, Herren S, Jakob S, Ayuni E, Mueller C, and Brunner T. 2004. "Intestinal Epithelial Cells Synthesize Glucocorticoids and Regulate T Cell Activation." J. Exp. Med. 200(12): 1635-1646.